

# Insubstantial charges of treason

**The confessions of a retired Russian spymaster make sensational charges of disloyalty against a generation of nuclear physicists now dead, and unable to answer for themselves.**

So Enrico Fermi was a traitor to his adopted country, the United States? That is what a former Soviet spymaster, Pavel Sudoplatov, is reported to believe in a book published last week (*Special Tasks*, Little Brown, \$22.95 & £18.99). So too were J. Robert Oppenheimer, the man with left-wing leanings who founded Los Alamos and brought the Manhattan Project to fruition, Leo Szilard, Fermi's friend at Columbia, George Gamow and Nils Bohr, the fountain-head of quantum theory in the 1920s. Not to mention, of course, Klaus Fuchs, a member of the Los Alamos team who was convicted of espionage in Britain after the Second World War. Sudoplatov tells a gripping tale, but an unconvincing one. Although much of what he has to say on other topics, the assassination of Trotsky for example, is interesting, his account of the doings of the Los Alamos traitors is so lacking in verisimilitude that it could well be a pack of lies.

Is it, or is it not? It would be easier to guess if it were clearer whether Sudoplatov is authentically the author of the inflammatory disclosures attributed to him. The introduction to the book, where the text is described as an "autobiography", is by Jerrold L. Schecter and Leona P. Schecter, a husband and wife. They explain that Sudoplatov was 85, and recovering from heart trouble, when they "began to interview" him in 1992. Jerrold Schecter is the one who arranged in 1970 for the publication in the West of Nikita Khrushchev's diaries, transcribed from tape recordings; that was not merely a publishing coup, but also a public service.

This latest Schecter venture seems more like a ghostwriters' job (but is nowhere explicitly described as such). The chief source is said to be an historical document compiled by Sudoplatov for his erstwhile employers, the KGB, filled out with 20 hours of "taped reminiscences" and with "notes and documents" provided by Sudoplatov's son, Anatoli. The title of the book derives from the intelligence unit of which the elder Sudoplatov was for several years the administrative head, and known as the "Administration for Special Tasks". The introduction says that Sudoplatov initialled the "most sensitive pages" of the manuscript now published.

## Ambitions

It is now, of course, no surprise that the former Soviet Union knew from an early stage that Britain first and then the United States nursed ambitions to make fission bombs from almost the time at which neutron-catalysed nuclear fission became known, in 1939 (see *Nature* **143**, 239; 1939). The

initial leak of supposedly top-secret information came from a British Foreign Office official, Donald Maclean, who fled Britain for Moscow on the eve of his arrest for espionage in 1955. To illustrate that point, the Schecters reproduce a KGB document dated 15 September 1941 detailing the proceedings of a British cabinet committee in London nine days earlier. Later documents detail meetings of the same or similar groups; the reasons why the British were persuaded by F. E. Simon and R. E. (now Sir Rudolf) Peierls that gaseous diffusion would be the best route to enriched uranium are fully recorded in messages to Moscow, for example.

## Proof

So is that not a proof that Sudoplatov has something special to say? Sadly, no. For these documents are taken in their entirety from an issue of the Russian-language periodical *Voprossi Istorii Estestvoznania i Tekhniki* which was circulated to Russian subscribers at the end of 1992 and afterwards withdrawn. Those with an interest in the Soviet development of nuclear weapons have been thoroughly familiar with these documents for more than a year. In the circumstances, it is over-sententious for the Schecters to say that two of the documents, which contain an account of how to make a nuclear bomb, have not been published in the public interest, but that all the material has been deposited in the library of the Hoover Institution at Stanford. That, as it happens, is where Edward Teller, Oppenheimer's chief rival in the years immediately after the Second World War, is a fellow.

Original evidence is otherwise scant. The Schecters have found (in the Moscow archive of the Ministry of Atomic Energy) some documents in which Igor Kurchatov, the head of the Soviet counter to the Manhattan Project, asks in September 1944 for answers by Soviet intelligence to specific questions about the progress of work at Los Alamos — was a reactor with natural uranium as fuel and ordinary water as moderator really feasible? Does a single plutonium fission really yield three spare neutrons? And so on. These documents are interesting, but nowhere is it suggested that they are Sudoplatov's contribution to *Special Tasks*. If the Schecters had really wished to convince their readers that their subject had original contributions to make to this tangled tale, would they not have reproduced some of his purported historical document for the KGB, and have deposited the full text of *that* in the library of the Hoover Institute? ➤



Another serious reason for doubting the value of this book is literary. Sudoplatov is a spy who led a dashing life, one year assassinating (with a bomb disguised as a box of chocolates) the emigré leader of Ukrainian nationalists in Paris, Konovalts, another keeping track of Soviet interests in Barcelona during the Spanish Civil War, yet another organizing the network of illegal operatives in Mexico which, after one false attempt, assassinated Trotsky. This part of the book reads like a spy novel — there is vivid detail and even personal emotion in among sentences of the utmost banality: for example, of Paris: “This was a city of history, and it occurred to me that the French Revolution lasted for 100 years, until the Paris Commune of 1871.” (Is that pure milk of Sudoplatov or of the Schecters?)

### Ambiguity

By contrast, the chapter labelled “Atomic Spies” is declarative in tone, and detail-free. It is also ambiguous in its choice of words. Thus “The most vital information for developing the first Soviet atomic bomb came from scientists developing the American atomic bomb at Los Alamos, New Mexico — Robert Oppenheimer, Enrico Fermi and Leo Szilard.” At that stage (in mid-1941), when Los Alamos had not been founded), there could hardly have been any other ultimate source. So the sentence may be literally correct, but misleading by calculated ambiguity. For what it is worth, the first enquiries (by Kurchatov) in the *Voprossi* documents about progress in the United States date from two years later, in mid-1943 (when Los Alamos was a going concern).

Soviet links with Oppenheimer are hardly more fully described. The first contact is said to have been one Gregory Kheifitz, the Soviet intelligence man at San Francisco, who led a double life as “Mr Brown”. Sudoplatov is reported to say that Kheifitz told him that he met Oppenheimer first at a party on 6 December 1941 to raise funds for refugees from the Spanish Civil War and that, at a lunch later in the month, Oppenheimer had “expressed his concern” that the “Nazis might succeed in building atomic weapons before the allies”; Oppenheimer is said also to have mentioned Einstein’s letter to President Franklin D. Roosevelt, sent but not published at that stage, urging serious consideration for the building of a bomb. (Fermi’s treason is no more clearly described; Kheifitz, who had moved to San Francisco from Rome, had in his earlier posting “targeted” both Fermi and Bruno Pontecorvo, one of Fermi’s then co-workers.) It goes without saying that none of these conversations would have been illegal or even improper at that stage.

### Infiltration

The nub of the Sudoplatov book recounts the strengthening of clandestine relations with Oppenheimer through the Zarubins, a husband-and-wife spy team based in the Washington embassy; Elizabeth Zarubin is said to have travelled often to Berkeley and to have infiltrated the Oppenheims’ circle of friends with the help of Kheifitz. Among other things, Oppenheimer is said to have taken her advice not to advertise his left-wing sympathies in order “not to call

attention to himself”, to have agreed to “share information” with “antifascists of German origin” and to have agreed to take Klaus Fuchs onto the payroll at Los Alamos. In a brief two pages, the book then describes how “Oppenheimer, together with Fermi and Szilard”, helped place moles at Los Alamos, Oak Ridge and Chicago and how, at a later stage, these people transmitted documents to their controller at the Washington embassy after being allowed access to them by “Oppenheimer, Fermi and Szilard, who were knowingly part of the scheme”. Gamow, the gregarious emigré physicist, was meanwhile blackmailed with threats against his family, still in Russia.

### Slander

This is strong meat. Those whose reputations are slandered in the book are no longer alive to defend themselves, but that does not mean that the issue can be buried, or even that there are none now alive who would like to see history rewritten. That is why the failure of Sudoplatov and the Schecters to produce a single document from any Moscow archive to suggest the quality of the information supposedly flowing like a torrent from Los Alamos is telling; then it would at least have been possible to tell whether Oppenheimer’s connivance in Fuchs’s admitted treason would have been necessary. (And would not Gamow’s revelations, even under duress, have been recognizable by his distinctive style?)

Similarly, it would have helped if Sudoplatov’s memory had run to a few reminiscences (gathered, perforce, from his ex-agents in the United States) about the ways in which material was dropped off at the illegals’ drug-store in Santa Fe, for example, or of the collection of information from Chicago. In the absence of indirect evidence of that kind, of course, there is literally no way of telling the difference between Sudoplatov’s account (as retailed by the Schecters) and a pack of lies. It is history of the worst kind; some will believe and others will reject the tale it tells, and there will be no evidence to arbitrate between them.

The sequel to the tale is now a legend. After leaving Los Alamos, Oppenheimer became the chairman of the Advisory Board of the US Atomic Energy Commission (AEC), and in that role argued for restraint in the development of thermonuclear weapons. In 1952, the AEC declined to renew his security clearance, against which decision Oppenheimer appealed. There followed a public hearing (in effect, a trial of Oppenheimer) which advertised to the whole world the intricacies of his ‘left-wing leanings’ and of his chance meetings with left-wing people. The AEC confirmed the decision while applauding the value of Oppenheimer’s previous public service. Senator Joseph McCarthy’s campaign against ‘communists’ was then in full swing. Oppenheimer returned to the Institute of Advanced Study at Princeton, where he died in 1967. The affair was a trauma for the research community in the United States. So that the bitterness of those times does not run over again, there are the strongest possible reasons why the research community should bend its energies to uncovering the truth, the Schecters and Pavel Sudoplatov notwithstanding. □



# US agencies split over inquiry into effects of radiation experiments

**Washington.** Deep divisions have emerged between the US Department of Health and Human Services and the Department of Energy over the value of an investigation into human radiation experiments in the United States that has been launched by the Clinton administration.

The \$3-million-a-year investigation will address public concern that has been heightened by Hazel O'Leary, the secretary of energy. She has been campaigning since December to "open up" the history of her department's predecessor, the Atomic Energy Commission (AEC) (see *Nature* 367, 303; 1994).

But Donna Shalala, the secretary of health, is known to be worried that the government-wide investigation will waste her officials' time, while fuelling public paranoia about all use of radiation in research and medicine.

The investigation is expected to yield

valuable lessons about scientific ethics, according to its chairwoman, Ruth Faden of Johns Hopkins University in Baltimore. But critics are concerned that the enquiry, set up by the president, manned by eminent lawyers and scientists, and supported by a strong full-time staff, will merely result in more public confusion and mistrust on the radiation testing issue.

At the first meeting of Faden's panel last week, the problems facing the investigation — such as the vast amount of data to be considered, and the ill-defined boundaries of the task — led some members to doubt whether it could be completed in a year as planned.

The investigation is to deal with all radiation experiments carried out on humans between 1946 and 1974, when the health department issued new guidelines to safeguard human subjects.

Shalala told the meeting that the Department of Health and Human Services, which provides \$11 billion for biomedical research annually through the National Institutes of Health (NIH), could provide a billion sheets of paper on radiation experiments if asked.

She also reminded the meeting that three-quarters of all NIH projects make use of radiation in some way. Like other government agencies, NIH awaits guidance from the new panel on exactly which radiation experiments it wants to know about.

Speaking at an informal lunch of Congress's Biomedical Research Caucus last Friday, the day after the inquiry was launched, Harold Varmus, the director of NIH, voiced his concern publicly. One aspect of his job that he did not like, he said, was devoting effort "at a time of scarce resources, to digging through a billion sheets of paper in the Clinical Centre about experiments we did in 1950".

In common with other government agen-

cies, NIH tried to avoid involvement in O'Leary's campaign when it started in December. Health department officials sought to draw a line between its health-related research and the then-AEC's interest in the effects of radiation from atomic bombs.

But as public interest rose, President Bill Clinton aligned himself with O'Leary and instructed all agencies to cooperate.

Faden says that the investigation will concentrate on three tasks, three duties and three themes. Its first task, she says, will be to establish what standards should be applied to radiation experiments during that period. Second, the investigation will review the experiments against that standard, and consider the need for follow-up studies, official apologies and financial compensation. Third, it will look at current experimental practices and policies.

The investigation's duties, Faden says, are to satisfy three constituencies: government that it can be trusted; the public that it can trust; and scientists that they will be treated fairly. The three themes that will run through the investigation, according to Faden, will be the ethics of research, the role of secrecy in a democracy, and the competing rights of the citizen and the state.

Welcoming the investigation, Senator John Glenn (Democrat, Ohio), chairman of the Senate's Governmental Affairs Committee, said it was likely that its report would lead to legislation to enable compensation of victims of radiation experiments. He added that the panel should run for more than a year if it felt this was necessary.

The panel will digest existing data on radiation testing, take evidence from the public and produce an interim report in six months that will include an estimated time of arrival for its final report.

**Colin Macilwain**



London. **Money can't buy you a title they say, but it could buy you the honour of naming this rare bird.** The charity BirdLife International is seeking donations towards preserving this new species of vireo, a small insectivorous bird discovered in the rainforest of the Colombian Andes in 1991.

Its discoverer, Paul Salaman, from the University of East Anglia, has waived his traditional right to have the bird named after him and it will now be named after the company or individual donating the most to its conservation. The as-yet nameless bird comes from the Choco region on the western slopes of the Andes and is thought to be unique to that region, where it has now been found at two separate sites.

Some 30,000 hectares of forest have been bought as a reserve in an area otherwise earmarked for development, and around £70,000 (US\$105,000) is needed towards establishing an endowment fund in conjunction with a Colombian foundation to run the reserve and ensure its survival. □

## 'Neglect at an end' for Irish science

**Munich.** The prospects for science in the Republic of Ireland took a turn for the better last week when the government announced that it is planning to resume funding for a strategic research programme which it abandoned last year.

This year, the government will make available £1 million (US\$1.42 million) for research grants in eight technological areas, including biotechnology, advanced manufacturing technology and advanced materials. In addition, it is making available £850,000 in basic research grants.

Scientists have cautiously welcomed the move as a possible signal of a change in

attitude towards science, whose fortunes sank to an all-time low last year when the government made available only £150,000 in non-medical grant money. Ireland has one of the lowest investments in research in Europe, and no real science policy. But a year-long campaign by university scientists has made research funding a hot political issue (see *Nature* 367, 586; 1994).

The prime minister, Albert Reynolds, admitted last week that the government had been slow to recognize the importance of science and technology. But he said that this "relative neglect" is now coming to an end.

**Alison Abbott**



# Hopes for space station fade on Capitol Hill

**Washington.** The prospects of the planned US space station surviving in Congress this summer darkened further last week, when the State Department failed to convince a key subcommittee that the administration's plan to build the station jointly with Russia makes sense either politically, economically or technically.

With only a month to go before the House of Representatives may get the chance to vote to kill the programme, congressmen on the House space subcommittee appeared dismayed at the lack of detail on the Russian deal presented by the State Department witnesses, James Collins.

Collins is a senior adviser to Strobe Talbot, the architect of President Bill Clinton's policy towards the states of the former Soviet Union. But committee member Martin Hoke (Republican, Ohio) told Collins that the hearing felt like a "pre-funeral" for the space station. "The administration has completely misunderstood the politics of this," said Hoke. "All you have to do is look at the numbers."

Last summer, the House voted by a majority of one to retain the station. Since then, some leading supporters of the project have publicly defected. "There is no groundswell of support for some form of 'new-age' relationship with the Russians," said Hoke, in a somewhat mocking reference to the administration's growing reputation for woolly and wishful thinking.

During testimony that congressional

aides say indicated a lack of direction at the State Department, Collins said that the space station was supported by the administration primarily as a "US flagship for technology and science". He said that the Russian partnership would make it cheaper, as well as assisting relations with Russia.

But many congressmen are angry that the partnership makes the project reliant on Russia. They point out that the United States was never prepared to depend on Japan, Canada or the European Space Agency for any critical part of the station, despite having worked successfully with these partners for decades.

Further concerns include a recent admission by Dan Goldin, the administrator of the National Aeronautics and Space Administration (NASA), that Russian participation will save the United States less money than was originally promised, and that Russia is already attempting to increase to \$650 million the \$400 million in US support promised for space collaboration in advance of the space station.

The space subcommittee and the Science Space and Technology Committee of which it is part do not have direct control over the space budget. But they have in the past been good friends of the space programme in general, and the station in particular; the lack of strong support on these committees therefore appears to bode ill for the project's survival.

George Brown (Democrat, California),

the chairman of the full committee, has already threatened to withdraw his support from the project if NASA's overall budget is not maintained at the level he considers necessary to cover both the space station and a strong science programme. But station supporters are even more alarmed by the certain defection of James Sensenbrenner (Republican, Wisconsin), the senior Republican on the space subcommittee.

Sensenbrenner is likely to carry a number of uncommitted Republicans with him. This would deprive the station of bipartisan support, and leave the Democrat leadership with the unenviable task of persuading more Democratic congressmen to vote for billions of dollars of expenditure to shore up Clinton's Russian policy, which is already in trouble elsewhere.

"If Clinton thinks that foreign policy aspects will make Congress appropriate \$17 billion for this he's got another think coming," said Sensenbrenner after hearing Collins' testimony, referring to the space station's projected total cost to completion. "Unless this deal is renegotiated to make this an American space station, the time has come for Congress to cut its losses."

A vote on the station is likely to take place in the House in late May or early June. If this is lost, the damage will probably be fatal — although the administration could spend the summer manoeuvring with the more supportive Senate to try to rescue the project.

**Colin Macilwain**

## Austria's new research head favours applied science

**Munich.** Austria should pay more attention to applied research, according to Arnold Schmidt, professor of physics at the Technical University in Vienna, who last month became president of Austria's science foundation, the Förderung der Wissenschaftlichen Forschung (FWF).

According to Schmidt, the quality of basic research has improved enormously over the past two decades, aided by increased government funding. But applied research has lagged seriously behind.

Schmidt has long been concerned with this problem. In 1988 he founded the Christian Doppler Society to link industry — which funds the society — and the academic world. The society now runs 16 small applied research laboratories, each costing around ÖS3 million (US\$250,000) a year.

The laboratories define their own research projects, but their research areas have been chosen after canvassing industry for its views on the type of research that it would find most relevant to its future needs, such as catalysis and environmental biotechnology.

Schmidt wants to bring a flavour of the Doppler Society to the FWF. Although the foundation is mostly concerned with basic research, a small part (ÖS50 million) of its ÖS700 million funds is provided by the Austrian National Bank and is earmarked for industry-orientated basic research. Schmidt hopes to use the same method of canvassing industry as he introduced in the Doppler Society for allocating this money.

"At the moment, a committee decides if a research proposal has industrial relevance, and in most cases that is as far as it goes," he says. Actual industrial needs should be taken into account far more, he argues.

In order to influence seriously the research structure in Austria, Schmidt wants the next government (elections are due in



**Schmidt: hoping for Doppler effect?**

October) to establish a series of applied research institutes, along the lines of Germany's successful Fraunhofer institutes. The principle has general support. But it will have more difficulty in gaining financial support, as research funds will be stretched once Austria joins the European Union (EU) next year.

The government has not yet decided if it will increase its total research investment to take into account the obligatory contribution it will be required to make to the EU's fourth framework programme. Negotiations are taking place to determine how much of this will come from Austria's normal research budget, and how much will be new money.

Schmidt hopes that the government will be generous. Like many in Austria, he believes that the country's poor applied research base means it will be unable to compete effectively for EU research funds, which are mostly industrially orientated. "Returns on [Austria's investment in EU programmes] will be low for at least the first few years," he says.

**Alison Abbott**

# UK sends double message in science spending plans

**London.** Despite the enthusiastic noises in last year's science white paper, total government support for civilian science and technology in the United Kingdom is expected to fall by more than 8 per cent over the next three years, according to figures published in London this week.

The biggest fall in spending will be in the Department of Trade and Industry (DTI). Following its decision to concentrate on direct support for technology transfer, the DTI's spending on science and technology is due to fall from £423.5 million in the current year to £271.2 million in the year 1996-97, in real terms a drop of 42 per cent.

In contrast, the Department of Health will see its research budget grow by 11 per cent in real terms. In line with policies that have already been announced, total spending by the six research councils remains virtually constant.

The figures are contained in the first edition of a new publication, *Forward Look*, which is to be put together annually by the Cabinet Office's Office of Science and Technology (OST). Based on individual departments' spending plans, the publication replaces the previous annual reviews of (past) expenditure on research and development.

According to the white paper, the preparation of the *Forward Look* is intended to allow the OST to check on the extent to which department's are fulfilling the government's commitments to steer science towards the twin goals of wealth creation and improving the quality of life.

"In its way, this document is almost as important as the white paper," says Bill Stewart, the government's chief scientific adviser, who was largely responsible for its

preparation. "This is the first time that different departments have put down in a single document their thoughts about their future spending on research."

*Forward Look* points out the government's success in boosting some areas of research, and in reining in expenditure in other areas. Spending on defence research, for example, is planned to fall "by about one third in real terms" by the end of the century.

Officials emphasize that two elements are likely to play a greater role in the preparation of similar documents in future years. One is the input from the recently-launched Technology Foresight exercise, expected to provide a consensus of the major fields in which government research spending needs to be concentrated. The second is the inclusion of a number of "output" measures which, says Stewart, will show how far departments have taken on board the strategy outlined in the white paper.

In his introduction to the document, William Waldegrave, the cabinet minister responsible for science, admits that, without such input, this year's document is "inevitably incomplete." Its main message in its current form, says Stewart, is to show how the promises to be made in the white paper are likely to be met.

But it is another promise — that was made last year by Prime Minister John Major in an address to the Parliamentary and Scientific Committee — which is also likely to be measured against the details of the *Forward Look* in future years. This was the commitment that the Conservative government would take steps to make good its neglect of science in the past.

That promise is likely to be measured, at least by the scientific community, primarily in financial terms. This year's document points out that the main reductions in government support for science are the result of cuts in defence and nuclear research. But the detailed figures also show that is not the whole story.

**David Dickson**



**Stewart: looking for co-ordination.**

## 'Broad approach' needed to innovation policy

**London.** An all-party Parliamentary committee has urged the government to broaden its efforts to stimulate innovation in industry away from merely concentrating on the health of the country's science base.

In a report published in London on Monday the House of Commons Science and Technology Committee says that the task of creating a climate in which innovation can flourish cannot be left to the OST alone, but needs additional support.

"It will not succeed unless all depart-

ments, including the Treasury, are seen to share the belief that the UK's future rests on world-class innovative industry," says the report. "Unless reforms are urgently undertaken, the United Kingdom will remain less able to exploit science and technology than many of its competitors."

In implicit criticism of recent retrenchment by the DTI, the committee says that the science base cannot provide all that industry needs "without abandoning some of its wider responsibilities". □

# Fermilab remains cautious on top quark 'discovery'

**Washington.** Scientists at the Fermi National Laboratory (Fermilab) in Illinois this week unveiled strong evidence for the existence of the elusive fundamental particle known as the top quark. But they stopped short of claiming its discovery.

The physicists say that the particle recorded by the Collider Detector at Fermilab (CDF) has a mass of  $174 \pm 17$  GeV, and that the statistical odds that the events pointing to its existence occurred by chance are 1-in-400.

"That may sound like a small probability, but for something as fundamental as the top quark, we'd want more statistical significance to claim discovery," says Bill Carithers of the Lawrence Berkeley Laboratory in California. Carithers and Mel Shochet of the University of Chicago are the elected chief spokesmen for the 440 scientists working on the CDF detector at the laboratory.

Carithers says that evidence collected about the top quark from 10 months of watching protons collide with antiprotons is "very, very consistent" with recent theoretical predictions of what the top quark should look like.

If its existence is confirmed, the particle — which has a mass 200 times that of a proton and decays in between  $10^{-25}$  seconds — joins bottom, up, down, strangeness and charm to complete the family of quarks predicted by the Standard Model of particle physics (see page 805).

Speaking from the office in Texas where he is now temporarily engaged closing down the Superconducting Super Collider, Fermilab director John Peoples says that he welcomed the breakthrough. But he declined to speculate on its likely impact on the fraught matter of high-energy physics funding. "I'm too naive to read the politics," said Peoples. "But from the scientific standpoint, this is good for the field." He added that the results would be useful to help work out how Fermilab's detectors needed to be upgraded to find out more about the top quark.

The CDF team, which represents 39 institutions in the United States, Italy, Canada, Taiwan and Japan, looked for two distinct modes of top quark decay in their study. With the first mode, they detected two events over the ten months, where 0.6 such events would be expected as "background".

Looking at the second mode of decay, the team found 6 events, against an expected background of 2.3, with one technique, and 7 against 3.1 with another. "When you combine these, the probability that the observed events were just a fluctuation is 1 in 400," says Carithers. The CDF team has submitted a 200-page paper on its results to *Physical Review D*.

**Colin Macilwain**



# Vaccine programmes facing threat of 'donor fatigue'

**London.** Global vaccination programmes responsible for preventing an estimated three million deaths a year from childhood diseases are in danger of losing funds provided by international donor agencies because of their success, according to scientists attending an international meeting at the London School of Hygiene and Tropical Medicine (LSHTM) last week.

Since 1974, the proportion of the world's children receiving a full course of the six major childhood vaccines has increased from less than 20 per cent to 80 per cent. But there is now concern that the very success of these immunization programmes may encourage donors to channel funds into other areas of public health-care instead.

The programmes receive money from the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP) and the World Bank.

According to Ralph Henderson, assistant director-general of WHO, sustaining the momentum of immunization programmes is crucial. "Five or 10 years ago, there was complacency about vaccines for preventable infectious diseases," he said at the meeting. "But immunization programmes have changed global epidemiology."

Henderson expressed particular concern that infectious organisms might at any time develop new strains resistant to current vaccines, to which the population would be highly susceptible. "We are sitting on a time bomb", he said, referring to the current levels of immunity to diphtheria in the adult population in Africa.

The campaign to eradicate polio from the Western Hemisphere, launched by the Pan American Health Organization in May 1985, was an attempt to revive donor interest by

creating new goals. But, although the campaign looks highly promising in that no cases of polio have been reported from the Americas for the past two years, the programme is turning out to be more expensive than initially thought. Henderson estimates that between \$1 and \$2 billion will be needed annually until the year 2000 to sustain it.

According to Felicity Cutts, the director of the conference and a senior lecturer at

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## Immunization is under threat.

LSHTM, the eradication of polio is feasible as long as the infrastructure for delivering the vaccine is in place. But in the lowest-income countries, which are mostly in sub-Saharan Africa, eradication cannot succeed, she claims, unless that infrastructure is first strengthened with the help of resources from the richer, developed countries.

The national coverage level of BCG (the current vaccine against tuberculosis) for children less than one year old in Cameroon, West Africa, fell from 95 per cent in 1990, following a universal children's immunization campaign, to 52 per cent in 1992. Peter Ndumbe, director of the Centre for Study and Control of Communicable Diseases at the University of Yaoundé, Cameroon, attributes this to "a new and infectious disease called 'donor fatigue'". **Laura Spinney**

# India seeks details of 'miracle' mix of microorganisms

**New Delhi.** Live microorganisms imported and released into the environment in violation of safety guidelines have created a furore among scientists in India. At the centre of the controversy is a private agency at Atami in Japan, known as the International Nature Farming Research Centre (INFAC).

Several acres of crop fields have been sprayed with a microbial culture "gifted" by INFRC to farmers with the promise that it will increase "crop yield, cut fertilizer bills, purify sewage, recycle kitchen garbage and even eliminate foul smell in cattle sheds".

According to the Japanese body, the culture contains "at least" 80 microorganisms beneficial to the soil. But it is keeping their identity and composition secret. INFRC plans to sell the preparation at \$2 a litre.

Many Indian scientists have been shocked by the ease with which the Japanese have inoculated Indian soil with strains of bacteria about which Indians know nothing. "We lap up anything made in Japan," says Balamani Bezbarua, a microbiologist at the Regional Research Laboratory in Assam. "But I didn't think we would go blindly for their bugs without knowing what they are."

Bezbarua makes up her own culture of microbes isolated from local soil, but is careful to treat crops only with the enzymes secreted by the bugs, and not the bugs themselves. "Release of exotic microbes in living form without knowing their identity is inviting disaster in the long run," she says.

The Department of Biotechnology (DBT), the custodian of safety standards in research in life sciences, claims that it was unaware that the Japanese preparation contained living microbes. Under DBT guidelines, release of imported organisms in living form needs approval from an expert committee that examines requests for such releases from Indian and foreign agencies. According to DBT, INFRC made no formal application.

INFRC staff provided the culture to their Indian "contacts" for distribution to farmers. A spokesman for INFRC said that when bottles were sent by post, the customs refused to clear them without authority.

INFRC also gave samples for field trials to individual scientists. One such trial at the Indian Agricultural Research Institute in New Delhi was stopped by its director, S. K. Sinha, who said it was going on without his knowledge.

INFRC claims that its product is made up of mixed cultures of naturally occurring species of organisms and is totally harmless. But Sinha says that there is still no justification for introducing the microbes to India without going through the proper channels.

**K. S. Jayaraman**

# Saltpetre dump blamed for Russian explosion

**Moscow.** A leading Russian expert on the physics of explosions has suggested that a mysterious series of major blasts three years ago in the small town of Savoso, 150 km from Moscow, was caused by the ignition of more than 30 tonnes of ammonium nitrate (Norway saltpetre).

The explosion, on 12 April 1991, left a large crater and generated wide-ranging rumours. Hypotheses on its origin in leading Russian papers included an earthquake, a meteorite impact or even radiation from an unidentified flying object.

There has been no official explanation. But a study carried out by Konstantin Shvedov, a laboratory director at the

Russian Academy of Sciences' Chemical Physics Institute in Moscow, concludes that it was caused by 32 tonnes of ammonium nitrate that had been stored there.

According to Shvedov, characteristics such as the crater size and shape, and the distribution of chemical products of the explosion, were similar to those of a well-studied incident in Oppau, Germany, in 1921. In addition, the impact on the environment corresponds to the total amount of ammoniac saltpetre known to be stored at the site.

Shvedov speculates that the explosion could have been triggered by a heavy object falling from a plane — or even by arson. **Carl Levitin**



# Price controls boost innovation in Japan . . .

**Tokyo.** Under a government policy aimed at curbing rapidly expanding medical expenditures, Japan's pharmaceutical companies have been required to accept significant cuts in the reimbursement prices for drugs under the national health system.

Ironically, however, the government's efforts to drive down prices are also intended to encourage greater innovation in drug development; and to some extent the policy is beginning to work.

Every two years, the Ministry of Health and Welfare revises the reimbursement prices for drugs paid by national health insurance, in order to bring them into line with the prices paid by medical institutions to pharmaceutical companies.

Japan is notorious for its failure to separate the prescription and dispensing of drugs. Medical institutions have long depended on the profits they can make by overprescribing drugs that they have bought at a significantly cheaper price than the official reimbursement price they receive from insurance (see *Nature* 342, 850; 1989).

In turn, pharmaceutical companies have tended to discount the prices of their drugs heavily to encourage hospitals to buy more. As a result, Japan has one of the highest per capita consumptions of prescription drugs in the world.

Interferon, one of Japan's leading biotechnology products with sales approaching US\$2 billion a year, has been hard hit in the latest round of drug price cuts by the Ministry of Health and Welfare (see below).

Sales of interferon began to boom in 1992, when the drug was approved for treatment of chronic hepatitis C, which is prevalent in Japan. Annual sales leapt from ¥30 billion in 1991 to ¥186 billion (US\$1.8 billion) last year (see *Nature* 362, 6; 1993).

The cuts in price range from 13.5 per cent for Takeda's Canferon-A, an interferon alpha product made using recombinant DNA technology, to 42.3 per cent for Mochida's interferon beta. They are expected to generate savings of more than ¥50 billion.

The cuts are unusually large and did not follow the ministry's standard price readjustment mechanism, which is based on the average selling price over a period of time. Rather, the ministry came under pressure from the Ministry of Finance to cut prices drastically because of booming sales.

There are, however, growing reports of side-effects for interferon alpha. Last year, there were several reports of pneumonia when the drug was used in combination with a Chinese herbal medicine also used for treating hepatitis C. Earlier this month, the Ministry of Health and Welfare reported 32 cases of attempted suicide, including 12 fatalities, thought to be a result of depression induced by the drug. **D. S.**

For the past several years, however, the ministry has been curbing this practice by monitoring the average actual prices paid and revising the official prices to close the gap between official and actual prices. In the latest round of price cuts, which took effect on 1 April, drug prices have been cut on average by 6.6 per cent.

The main aim of the cuts is to hold down Japan's rapidly rising medical expenditure. This has soared to about ¥25,000 billion (US\$240 billion) a year, and is expected to

rise even faster with the rapid ageing of Japanese society.

But a secondary aim is to encourage companies to develop innovative drugs, on the basis that novel drugs with no competition will be able to sustain higher prices in the market. And the ministry does deliberately set higher prices for new drugs.

At first, the policy produced the opposite effect from that intended. In the 1980s, manufacturers produced many 'new' drugs that were merely modifications of existing compounds, with little if any additional benefit. But leaders in the industry now realize that the only way to beat the price-cutting system is to produce innovative drugs that will not be subject to discounts.

One of the first to make a move was Yamanouchi Pharmaceutical. Under the leadership of its new vice-president for research and development, Teruhisa Noguchi, the company established a new institute of molecular medicine at its research centre in Tsukuba science city in 1991. It has also set up a research institute in the United Kingdom near the University of Oxford to carry out research in molecular and cell biology related to drug development (see *Nature* 361, 764; 1993).

But Japan's pharmaceutical research still remains weak compared with that of other advanced nations. Takeda spends only a little over ¥60 billion (\$600 million) a year on research and development, about half the research budget of companies such as Roche of Switzerland or Glaxo of the United Kingdom. Furthermore, only a handful of Japan's drug companies have established research operations overseas.

The squeeze on drug prices is holding down profits, and although the drug industry has weathered the recession better than most of Japanese industry, the industry's annual increases in outlay for research and development are beginning to decline from about 10 per cent a few years ago to around 5 per cent in 1993. **David Swinbanks**

## ...but are opposed in US Congress

**Washington.** To the relief of the US biotechnology industry, the new-found opposition of a key lawmaker appears to have killed efforts by the Clinton administration to set up a mechanism for the federal review of the initial prices of 'breakthrough' drugs as part of its health-care reform plan.

Last week, Representative John Dingell (Democrat, Michigan), chairman of the powerful House of Representatives Energy and Commerce Committee and a central player in the health-care reform debate, agreed to delete a clause from any health-care reform legislation establishing the reforms that would have set up an advisory council on breakthrough drugs.

Industry representatives have long argued that the mere spectre of heavy-handed regulation, seen as a mechanism for some form of indirect price controls on new products, is frightening away investors in an industry that relies heavily on investor capital. They also claim that the prospect of such regulation is already depressing stock prices and causing companies to cut back on research and development (see *Nature* 368, 180; 1994).

In agreeing to drop the provision, Dingell was responding to pleas from two freshman members of Congress, Representatives Lynn

Schenk (Democrat, California) and Marjorie Margolies-Mezvinsky (Democrat, Pennsylvania). Both Schenk and Margolies-Mezvinsky hold crucial votes on his committee, and Dingell agreed to make the concession in the hope of winning their support for some of the broader reforms being sought by the Clinton administration as part of its health-care reform plan.

In a letter to Schenk, whose congressional district of San Diego contains about a hundred biotechnology companies, Dingell acknowledged that to single out new or 'breakthrough' drugs for draconian price regulation "is not a good idea" and "should not be included as part of any comprehensive health-care reform".

Margolies-Mezvinsky has obtained a similar commitment from Dingell to modify a provision in the administration's health-care reform plan giving the secretary of Health and Human Services the authority to negotiate special rebates or discounts on new drugs paid for under Medicare.

In cases where agreement could not be reached with the drug manufacturer, the secretary would have been able to 'blacklist' the drug, excluding it from reimbursement under the government's Medicare programme. **Diane Gershon**

# Astronomers in Europe urge new Hubble pact

**Paris.** European astronomers are putting pressure on the European Space Agency (ESA) to move quickly to extend an agreement with the US National Aeronautics and Space Administration (NASA) guaranteeing them observing time on the Hubble Space Telescope when the existing agreement expires in 2001.

They are worried that, if such action is not taken promptly, they may lose some or all of their access to the telescope in 2001. But ESA is concerned that committing new funds to Hubble in the near future could mean reducing support for other space science projects.

Under the current agreement, European astronomers are guaranteed a minimum of 15 per cent of observing time on the telescope, which was launched in 1990. (In practice, they win around one-fifth, based on competition for scientific proposals.) In return for viewing time, ESA has supplied two sets of solar panels and the Faint Object Camera (FOC). It has also provided 15 members of staff at the Space Telescope Science Institute in Baltimore. ESA officials admit that "[w]e get a very good deal".

But Europe's astronomers say that this honeymoon could end abruptly in 2001 if ESA does not renew its dowry in good time. Indeed, NASA officials say that, if ESA delays commitment too long, it may be "too late"; they also say that financial pressure on the US agency may force it to change its policy of giving other countries such as Australia — the third most frequent user of Hubble — free access to the telescope.

ESA officials say that the astronomers' warning has been registered by the board of the agency, which formally discussed the

issue with NASA for the first time at a meeting last month. Giacomo Cavallo, the head of ESA's science programme coordination and planning office, is keen to reassure astronomers. He says that "ESA is committed to Hubble", and is actively looking at ways to ensure continued participation beyond 2001.

But many astronomers remain disappointed at the likelihood that towards the end of the decade the agency will limit itself to contributing to the operational costs of the telescope, rather than taking up a proposal to develop jointly with NASA the Advanced Camera, due to be fitted during a servicing mission in 1999 (see *Nature* **368**, 573; 1994).

By that date, the FOC will have been in orbit for nine years, and the Wide Field and Planetary Camera for five, well beyond their design lifetime. ESA officials say the Advanced Camera "would let us go two magnitudes fainter than the FOC, with a

field of view four times bigger".

Joining up with NASA would make it possible to double the proposed budget for the camera to around US\$100 million, and provide a 'superior' instrument to that now planned.

According to NASA scientists, however, ESA representatives at last month's meeting "made it very clear that they were not interested" in helping to develop the camera. Cavallo says the main obstacle is that ESA would have to find US\$50 million immediately, as the camera would have to be built within three years, and that this would mean delaying other projects.

But Cavallo adds that even if ESA cannot take part in building the Advanced Camera, European astronomers will still be free to participate directly. He says that ESA will encourage NASA to seek help from Europe's national space agencies, and might be in a position to make a small additional contribution to such efforts. **Declan Butler**

## Spain likely to beg poverty at CERN

**Munich.** Spain is threatening to cast a shadow over next month's expected approval of the Large Hadron Collider (LHC) at the European Laboratory for Particle Physics (CERN) by continuing to demand a significant reduction in its annual contributions to the organization.

Spain has withheld its subscription to CERN for the past two years on the grounds that it wants a better return on its investment, and it now owes the laboratory SFr130 million (US\$90 million). Earlier this month, it asked for more time to consider a compromise offer of a 20 per cent discount in its subscription over the next five years, having originally asked for a 30 per cent reduction.

But Spain's actions have annoyed some of CERN's major member states, which argue that it has reaped significant political advantages from its decision to join CERN in the 1980s — a prelude to becoming a full member of the then European Economic Community — and that the amount of money in dispute is relatively small compared with the economic benefits it has obtained.

Contributions from CERN's 19 member states are based on gross national product (GNP), averaged over the previous three years. Spain's economic spurt during the 1980s meant that its subscription grew by nearly 50 per cent between 1989 and 1993. It now pays around 8 per cent of CERN's total budget, making it the fifth biggest contributor.

But Spain's economic boom was short lived, and the country is now deep in recession. Furthermore, Spanish officials complain that, when measured against financial

contributions, Spain has the lowest number of staff and visiting scientists at CERN, and wins less than 1 per cent of the total value of industrial contracts issued each year.

The dispute seemed near resolution earlier this month when CERN council members were asked to consider a proposal from Christopher Llewellyn-Smith, the laboratory's director-general, and Hubert Curien, the president of its council, following negotiations with Spanish delegates and government officials in Madrid.

Under this proposal, Spain would repay its outstanding debt immediately by underwriting a loan taken out by CERN, and in return would get a discount on its contributions over the next five years. Reductions would be on a sliding scale, starting with 40 per cent this year when economic problems are particularly hard, and averaging 20 per cent reduction over five years.

Elias Fereres, the secretary of state for universities and research, says that the two sides are close, and hopes for a quick resolution. But Fereres has limited influence over the decision. Although his ministry is responsible for Spain's scientific activities in CERN, for historical reasons the budget is controlled by the ministry of industry, which has little interest in fundamental science, while the ministry of finance, which ultimately holds the purse-strings, is taking a very tough line.

Llewellyn-Smith wants the issue resolved before CERN's council meeting in June, when member states will vote — it is hoped unanimously — on the approval of LHC.

**Alison Abbott**

## ESA plans 'go it alone' collaboration strategy

**Paris.** The European Space Agency (ESA), upset at the way in which foreign partners have vacillated over its participation in collaborative projects, has decided that all future missions will be designed so that foreign participation is not essential to achieving their main scientific goals.

Last year, ESA decided to go ahead with a scaled-back version of the first mission (Rosetta) to land instruments on the surface of a comet, without the planned involvement of NASA. This decision was taken after the US agency had indicated that funding for its participation would probably not materialize (see *Nature* **365**, 681; 1993).

ESA has now formally decided to design all missions so that they can be launched and achieve their main scientific goals even if partners pull out. **D. B.**



# Stanford falls in line on conflict of interest rules

**San Francisco.** Stanford University in California has rejected the advice of an advisory panel set up by its Office of Technology Licensing and changed its rules on intellectual property rights, making it no longer possible for individual faculty members to retain the rights to their inventions.

It has also resolved a contentious dispute over computer software by deciding that the university retains the rights to patented software, but that ownership of all copyrighted material will remain with the inventor. Both changes are part of a broad range of new conflict-of-interest rules approved by the university Senate on 14 April.

Stanford has been one of the most successful US universities in generating income through the successful exploitation of university-based inventions; last year it earned over \$25 million in this way.

The new rules emerged from two years of study by a committee chaired by Craig Heller,

a policy generated conflicts of interest by putting faculty members in the position of deciding which source of support led to a particular invention. The university Senate has now decided to fall in line with most other US universities and to stipulate in its new rules that all discoveries will now remain the property of the university.

The new policy also requires professors to be frequently on campus and to commit their scholarly expertise, efforts and research primarily to Stanford. The provisions would prohibit professors from serving in a managerial role at companies and from acting as a principal investigator for research that could be done at Stanford.

Faculty members are allowed to own stock in outside companies and to sit on corporate boards of directors. In addition, deans at Stanford would be able to designate certain conflicts of interest as manageable.

Several elements of the policy anticipate guidelines expected to be published shortly by the National Institutes of Health and the National Science Foundation requiring institutional oversight of faculty financial relationships.

But the guidelines fall short of strict provisions at universities such as Harvard, which require advance approval for most financial connections between professors and commercial enterprises.

The new guidelines have been accepted because most faculty members recognized that universities were attracting outside scrutiny of their financial ties, and that there was a need for stronger rules at Stanford, according to Barton Bernstein, professor of history and a member of the committee.

"We didn't have rules to effectively guide faculty and to protect the university," says Bernstein, who argues that the intellectual property created by a university faculty member should be social property. "We didn't know whether egregious things were going on."

Charles Kruger, ex-officio dean of research who is also on the committee, said the guidelines would be unlikely to change existing outside relationships among faculty. But he said that they might limit gifts or other contributions from companies in which professors have a financial interest.

Katharine Ku, director of Stanford's Office of Technology Licensing, said it was unclear how significant the impact of the policy — which committee members stress is not aimed at increasing university revenue — is likely to be on university income. She praised the change and said she did not feel that it would discourage faculty from encouraging the exploitation of their inventions.

**Sally Lehman**

# France backs off strict limits on embryo research

**Paris.** By adding amendments to a proposed new law on bioethics, the French National Assembly has toned down harsh restrictions on research using human embryos and related aspects of human fertility which the Senate had voted for earlier this year.

Last week, the Assembly adopted by a large majority three separate bills that will make up the new law. It was the second reading of the bills by the Assembly, which was packed for the vote. The bills will now return to the Senate for a second reading, before becoming law.

The adopted bills would ban eugenics, genetic testing (except for research, medical or judicial purposes), surrogate motherhood and patenting of parts of the human body. The law would also restrict *in vitro* fertilization to living sterile couples of reproductive age. The bills would permit somatic gene therapy, but not germ-line therapy. Infringements would carry a fine of up to FF92 million (US\$340,000), or prison.

All this has already been approved by the Senate. But the Assembly modified the senators' wish for a complete ban on pre-implantation diagnosis and selection of embryos. In line with the opinion of many researchers and physicians (see *Nature* 367, 209; 1994), it voted that such techniques should be permitted in "exceptional cases" where parents risked bearing a child with a "particularly serious" genetic disease.

Similarly, the Assembly subtly changed the Senate's ban on all embryo research that threatens the "integrity" of the embryo. Although it maintained the condition that the embryo should not be harmed, it stated that such research should be permitted for medical purposes; in practice, this would, for example, allow cells to be removed.

The Assembly also rejected both a requirement that embryos be implanted within eight days of fertilization, and a ban on the destruction of spare embryos — of which there are around 20,000 in France.

The bioethics bills would also amend the data protection act to allow physicians to provide biomedical researchers with confidential information about patients provided that patients agree and that their identity is concealed. Such data may not be used for international projects, unless the other countries involved have equivalent legislation.

Researchers wishing to use such data would have to apply to a new commission, appointed by the Ministry of Research and Higher Education, and the Commission Nationale de l'Informatique et des Libertés (CNIL). To speed up the process, the ministry would be required to make a ruling within one month, and the CNIL within two.

**Declan Butler**



professor of biological sciences, to define faculty responsibility and pinpoint situations that compromise integrity.

Until now, a conflict-of-interest policy dating from 1966 had focused primarily on setting limits on the hours professors could engage in outside consultancy or other income-generating activities. But professors retained all intellectual property rights.

An early draft of the new rules allowed faculty to retain title to their inventions unless a government grant stated otherwise. The committee said at the time that it believed that the opportunity for entrepreneurship and financial reward helped to attract top researchers and promoted technology transfer to the commercial sector.

The licensing office's advisory panel had argued that giving individual scientists title to their inventions acted as a powerful incentive to them to seek ways of exploiting the results of their work.

But the committee later decided that such



# Understanding religion

SIR — May I set out the prolegomena for any consideration of the religion–science relationship?

First, the obvious distinction must be made between scientific method and scientific cosmology. If we accept scientific method as the most refined and sophisticated use of our intellectual capabilities, then of course the study of religion must be as subject to its discipline as any accepted branch of science. The antithesis between science and faith is based on a misunderstanding.

Second, certain aspects of our mind must in some way be excluded from the strict causality embraced by all traditional scientific disciplines (including modern statistical versions of the older atomic cosmology). This is what I understand to have been proved by Gödel's theorem, although, if my understanding is challenged, I need only fall back on the old paradox that if strict causality rules, then this applies to present discussions, and scientific objectivity has no meaning. This is the paradox to which I believe Gödel gave mathematical expression. It is also for this reason that hypotheses such as that of Mario Vaneechoutte (*Nature* 365, 290; 1993) may contain truth but not the whole truth.

Third, the scientific study of the phenomena linked to 'religion' requires, like any other scientific discipline, years of both theoretical and practical study. Until we have embarked on this, our criticisms can be only at the level of the character in the Aldous Huxley story who happily refuted the theory of relativity on the basis that there is no room for a fourth right angle.

Bertrand Russell, when asked what he would say if, when he died, he found that there was after all a God, replied that he would ask: "Why did you not give us the data, Lord?" But the data are there, and no more esoteric than, say those of astrophysics or molecular biology to an 18-year-old student studying sociology.

One problem in approaching the subject is the missionary zeal of all 'religious' denominations, whose main technique has always been to inculcate guilt. Because their overlay of competing dogmas presents such an easy target through which to defend ourselves from guilt feelings (some of which derive from childhood education), the effect is to hinder rather than to promote understanding.

Some of us may be without the capacity (which may correlate imperfectly with measures of IQ) and others without the drive and perseverance to embark on the journey towards religious understanding. But those who might make the attempt should not be deterred by criticism arising from ignorance but presented under the

guise of science. What was said centuries ago is worth repeating: that counterfeit coin can circulate only because real money exists.

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SIR — If our brains had not given us a fairly reliable picture of the world as it really is, we would probably have been eliminated by natural selection in favour of a species possessing one that did.

It is therefore disturbing to reflect on the paradox outlined by R. H. Good (*Nature* 366, 296; 1993) arguing — to my mind convincingly — that holding an almost certainly false religious belief confers a decided biological advantage in inter-tribal conflict. However, fortunately for us, it is probable that this advantage is overborne by the technological superiority gained by those who subscribe to scientific principles: for example, the West's development of the atom bomb more than compensated for its lack of belief in a God Emperor in bringing the Second World War to an end.

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SIR — Friedrich Katscher (*Nature* 367, 677; 1994) asks if there is scientific proof that Jesus turned water into wine and, of course, there isn't. No tangible evidence remains from that miracle and no scientific investigation is possible. The lack of tangible evidence is a major problem for those wishing to subject the biblical miracles to scientific investigation.

However, all is not lost, for the Bible claims that God also used his supernatural powers miraculously to create matter, life and consciousness, and these are everywhere abundant. If they were truly created supernaturally, then it is reasonable to assume that their origins lie outside the scope of science to explain and the failure of science is predicted in this respect. The investigation of that prediction provides a powerful negative test of the truth of biblical miracles.

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SIR — I think perhaps M. Hammerton's zeal (*Nature* 367, 677; 1994) has somewhat clouded his logic.

Whatever one may think of the truth, or otherwise, of creationism, it remains true that thousands of professional scientists worldwide profess creationist beliefs. This

horrifies some and delights others, but the point is that the same could hardly be said of belief in a flat Earth, or the idea that cattle disease is the result of sorcery.

Besides, guilt by association is not a valid argument against creationism, or evolution for that matter — particularly when the association is, as in this case, spurious.

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SIR — In his article "Language for a polyglot readership" (*Nature* 359, 475; 1992), John Maddox raised the question of whether biblical and other Western cultural references should be avoided for non-European readers. My answer is no, as allusive writing is fun to read and motivates the reader to improve his or her understanding of the cultural side of the English language. On the other hand, it would be nice if people writing in *Nature* showed the same interest in foreign culture, and avoided the kind of comments published in "Praying for baby hamster's souls" (*Nature* 368, 486; 1994). Suggesting that the feeling of guilt is the reason for praying is at best a lack of curiosity. Moreover, as an agnostic, I find that giving a soul to a dead animal is as rational as giving one to a dead human. So why is *Nature* not making fun of Western prayers? For once, a British journal has confused humour with irony.

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SIR — The recent column by John Maddox "Defending science against anti-science" (*Nature* 368, 185; 1994) seems to reduce science to a proselytizing political ideology. Science, as a way-of-knowing, is not furthered by witch-hunting, rooting out of 'non-believers' and paranoia about other ways-of-knowing that challenge the received wisdom of the scientific establishment. It is wrong-headed to ask whether science should tolerate non-science. Simply put, there is no place for Torquemada in science. Science, at its best, relies on a free and curious spirit of inquiry, open methods, a readiness to admit and correct error and a sincere aversion to dogmatism. It questions everything (including its own processes), and avoids the Scylla and Charybdis of arrogance and partisanship, the sure death of the open mind. Science will make more progress by continuing its noble work, confident in its own abilities, rather than by defensive posturing against any perceived threats to its turf or its achievements.

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# Mexico's bid to join the World

**The 80 million people of Mexico are looking to 1994 (and NAFTA) to signal full membership of the international community. The assassination last month of a presidential candidate is a set-back, but need not be a huge one. This survey of science in Mexico shows that the foundations have been laid for continued improvement.**

MEXICO is not your ordinary developing country, a mass of impoverishment trapped in a sea of insoluble problems. On the contrary, it is made up of an exuberant and often sophisticated people embedded in an ancient setting, both anthropologically and tectonically. Mexico is attempting to leapfrog its way to 1980s-style prosperity while turning its monuments and topography into its heritage. And Mexico has more going for it than its partners in the recently negotiated North American Free Trade Agreement (NAFTA), Canada and the United States, can have realized when they were disdainfully negotiating that deal.

This survey of science in Mexico can be taken as a celebration of that goal. Of course, Mexico is not alone among newly awakened countries in believing that science and technology, or R&D, are one of the important means by which its ambitions can be realized. China is obviously one. So (see *Nature* 366, 611-625, 1993) is India. So, at the other end of the scale in size, is Quebec (not yet, and perhaps never to be, a country on its own) (see *Nature* 368, 389-394, 1994). But Mexico's way of bootstrapping its way into the future is novel and imaginative. It would be good for everybody if it can succeed.

These ambitions are in part a consequence of the serious fright that Mexico's politicians were given in the mid-1980s, when for a time (in 1982) it seemed that the country would be bankrupt. What went wrong is that the oil industry, built up during the 1960s and 1970s on the strength of reserves in and around the Gulf of Mexico, and run as a nationalized industry, was caught out by the failure of the price of oil to continue to rise.

The government, which had been borrowing abroad on the strength of the expected revenues, was left with a debt of US\$80 billion in round numbers. The cost of servicing the debt became insupportable, causing a domestic crisis (unemploy-

ment and high inflation). Mexico also became the first (but not only) cause of the crisis which then hit the international commercial banks, which were forced to set about writing down their supposed assets.

One of Mexico's responses was to denationalize many state-owned industries (banking, for example) and to deregulate what had been a tightly controlled economy (in which, for example, nothing could be imported without a licence). Mexico has won applause for the daring of those steps. President Carlos Salinas, in office until August's election, is forever saying that Mexico will never make the same mistake again, whence the powerful political commitment to investment in science and technology.

In that connection, the timing of this survey is both deliberate and unfortunate. Two of us (see box) spent three man-weeks in March interviewing government officials, academics and government employees, mostly in research laboratories. That, it seemed, would provide an account of Mexican science as it is now against which to measure the changes the election would bring.

But soon after the second of us left the country, the government party's presidential candidate for the election to be held on 21 August, Mr Luis Donaldo Colosio, was assassinated on 23 March at Tijuana, just south of the border with California. First impressions (gathered by telephone) that the assassination will make no difference to the outcome are believable. But whether the social condition of Mexico will be unchanged, say a year from now, is another matter.

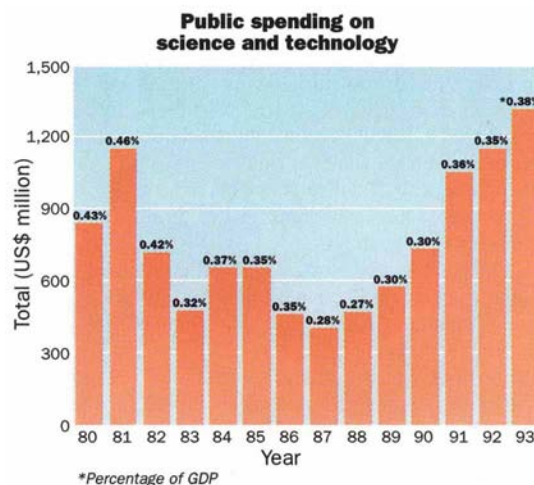
That is one internal source of pressure on the status quo. Another is the armed (but mostly bloodless) rebellion against the civil authorities, in the south-eastern state of Chiapas, by people of largely Amerindian stock. Nobody is clear exactly what the rebels want (more money, more land or more freedom?); the government has temporarily quelled the troubles with promises. Implementing them will be neither simple nor cost-free.

Of the two developments, the assassination casts the longer shadow: Mexico's studied sophistication may not be as durable as it seems. The aftermath of the

assassination could even (after the election) split the Institutional Revolutionary Party (PRI), which has provided Mexico with its presidents for the past 65 years. At least in principle, that could dilute the political commitment to research.

Luckily, there are countervailing influences, of which the chief is NAFTA. President Bill Clinton in the United States may look back on his battle last year to persuade the US Congress to endorse the treaty as his first real legislative victory, but for Salinas it is politically a much more valuable prize; a proof to 80 million people that Mexico is now part of the modern world. To prove the point, Mexico joined the Organization for Economic Cooperation and Development (OECD) on 14 April.

What will be NAFTA's consequences for science in Mexico? NAFTA is not the



**The dip in investment in the 1980s coincided with low oil prices**

Treaty of Rome, on which the European Union is founded, but merely what its name says, a free-trade agreement; mutual tariffs with the United States and Canada will be reduced to zero over the next five years, but there are no arrangements for supranational legislation to ensure a level playing field for competing companies, nor is the free movement of labour a condition of the deal. Illegal Mexican immigrants in California will still be illegal.

Even so, NAFTA will matter, and not just by helping Mexican researchers more easily to import equipment from North America (see page 799). Mexico expects that the demand for technical skill will

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quickly increase as indigenous companies seek to export sophisticated products or even are acquired by new owners from the North. (But Mexican agriculture may yet overturn that calculation.) The trouble is that there are not enough people — trained people, that is.

That is why everybody who talks about public policy and science talks first about the shortage of qualified scientists and engineers: by one definition there are between 6,000 and 7,000 of them. Luckily, the usual developing-country problem of inadequate equipment and infrastructure may have been substantially (if only temporarily) solved by the loans from the Inter-American Development Bank (IDB) in 1991 and from the World Bank last year.

So Mexico's research establishment has mounted a campaign to recruit people from elsewhere, both expatriate Mexicans and people from Central Europe and Russia (see page 793). The numbers involved are small (a few hundreds) and the inducements are substantial. But that unusual import trade in skill on two legs could be influential in the long run.

So could be the new and tidy administrative brooms sweeping through government offices — at least those administering research. The people in charge of programmes are young and well-qualified, the kinds of people journalists describe as technocrats. And transparency (to Mexicans as well as visitors) has

become almost a fetish, which is important in a country where the patronage of public servants is traditional, habitual and profitable (for the public servants). (In 1991, 12,000 public officials were sanctioned for administrative misdemeanours and N\$300 million was recovered in the form of penalties and forfeits.)

In such a climate, who can be sure that research grants go to the best qualified researchers? The research council CONACYT has one answer: publish the outcome of the two annual grant-competitions in the newspapers, as advertisements. In the same spirit, the membership of the committees that adjudicate on the eligibility of researchers for membership of one of the four categories of the SNI (Sistema Nacional de Investigadores) are publicly known, as are the criteria they use in their assessments (citation indices count).

The allocation of funds between government research laboratories is even more transparent; there is a piece of algebra involving four parameters from which anybody with access to the data and a knowledge of the total budget could calculate the amount due to a laboratory. Whatever may be the frailties of the rest of the public administration, the administration of science in Mexico is fair, or at least transparent, and has been for the past two or three years. It is also economical. The director-general of CONACYT, Mr Fausto Alzati, boasts of the annual rent he

has saved by moving from a quasi-palace to a modern office block (and shedding two-thirds of the previous staff in the process).

That is a remarkable achievement, but an administrative one. To exorcise nepotism is an invaluable preparation for the future, but the future is not yet here. General science-based prosperity has not yet begun to flow. How, in the meantime, is all that spending (the government's share was the equivalent of US\$1 billion, or 0.36 per cent of gross domestic product, in 1991) to be justified? The danger is that the next cohort of politicians will fail to share the patience of Salinas's advisors, who unanimously affirm that there is only one present objective: finding more, and more able, researchers. They shake their heads in disappointment when they acknowledge that the numbers are not growing all that quickly.

These are early days. Mexico's present arrangements for research are creatures of the Salinas presidency, and mostly of the second half of Salinas's six-year term. And it is true that there is an evident shortage, among researchers, of people who talk about their fields of interest with that unmistakable certainty (which may, of course, be mistaken) that comes from understanding what the rest of the world is up to and from guessing where it may be wrong. Too many of Mexico's research projects are other people's figure legends writ large, but differently applied.

Therefore, sooner rather than later, the government of Mexico and CONACYT, which is designated by its enabling law of 1970 to call the shots, should turn their attention to more radical rearrangements of the present system. Here are some obvious ways in which that should be done, arranged ontologically, in the order in which a person's career would be affected. The brashness of what follows should not be mistaken for a lack of understanding of the impediments to change.

**Preparatory education.** Faculty members at the Universidad Nacional Autónoma de México (UNAM, see page 794) are divided on the propriety of providing secondary education for intending students — some see it as a challenge and an opportunity, but most are bored. But the practice has now spread to state universities. It should be stopped, if gradually. Researcher-academics do not famously appreciate the needs of younger teenagers (whom they would not deign to teach), but are at present saddled with a responsibility they cannot possibly discharge. But the system, being regional, militates against the nation-wide meritocracy to which Mexico aspires.

**Decentralization.** Historically, UNAM has been the chief provider of higher education in Mexico, and virtually the sole public research laboratory as well. By any academic standards, its 230,000 adherents

(staff as well as students) ensure that it is conspicuous; even now, after sporadic attempts at decentralization, UNAM employs nearly a quarter of those qualified to belong to SNI and spends a fifth of central government's research budget.

The UNAM model, valuable though it may have been a century ago, now has several demonstrable drawbacks: researchers hardly ever teach classes of undergraduates, while teachers of undergraduates are not expected to be competent at research. UNAM is also too big to be manageable in terms that make sense to researchers seeking to tilt at an internationally noticeable windmill; if one research institute or another devised a really outstanding project, it would have to wait for years before it knew that the university (whatever that might be) approved. UNAM is also inefficient at its main task of graduating people with high skill. As at many other metropolitan universities (Paris, for example), UNAM is not a good at getting graduate students through their courses quickly. It is not merely that the world's biggest city offers a multitude of distractions, but that graduate students must forever calculate whether the effort spent in writing a thesis will be worthwhile; mid-career scientists in Mexico have the hardest time. Especially because graduate education is free (and most graduate students are paid a small stipend), there is a case for meeting the national need for people more expeditiously by

enforcing some kind of contract of mutual understanding between students and their supervisors.

To suggest that UNAM, Mexico's academic pride, should be radically changed is not the philistine proposal it may seem. Groups of researchers (not individuals) could (and should) be offered the choice of shouldering some responsibility for teaching at UNAM or at a state university. Or they might exempt themselves from menial work with undergraduates by taking responsibility for training graduate students in a reasonable length of time. That would not solve the national problem, but it would be part of a solution.

**Mid-career money.** The most serious obstacle to the improvement of science in Mexico is the enforced deprivation of people newly graduated with a PhD. Their best strategy is to work abroad for as long as it takes to acquire a reputation, and a ranking on the SNI scale (by which time they are likely to be permanently welcome where they already are). The starting salary for an academic (without SNI or university uplift) is N\$1,640 a month. That is not enough to run a car of any kind, or to rent a decent apartment in Mexico City.

More senior academics say that this financial bottleneck should not matter; senior people are well able to earn the equivalent of more than US\$1,000 a month, with SNI and university bonuses added to their basic pay. But that is tantamount to asking that younger academics should help to make a prosperous future for Mexico without sharing even in present prosperity themselves. It is no wonder that the younger people stay abroad.

**The bureaucracy.** The administration of science has been amazingly improved. People agree on that even when they shake their heads over the future. The reforms of the early 1990s were a substantial part of the reason why the World Bank agreed in 1991 to lend the government of Mexico \$187 million over four years; the bank, in its document on the project, is especially approving of the way in which research projects are now being evaluated by peer review, with non-Mexicans involved both as reviewers of grant proposals and as members of the evaluation committees.

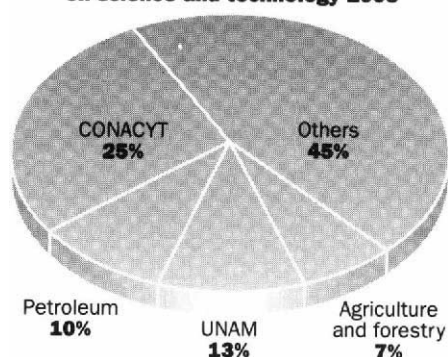
CONACYT is the centrepiece of the new arrangements, serving both as a mandatory source of advice to the government and as a grant-making organization. For the time being, its customers seem content. But it is inevitable, some years down the new road, that this dual role will create a kind of conflict of interest. At the same time, the council's responsibility for the whole range of basic research (CONACYT spans the social and the natural sciences as well as much applied research) will not indefinitely be easily accomo-

dated beneath one umbrella.

Not now, but later, there will be a case for giving the disciplinary evaluation committees more autonomy. Then or even later, CONACYT's responsibility for strategic planning could well become the organizing principle of a ministry of science and technology.

**Public spending.** If the government of Mexico is serious in its protestations about the importance of science and technology,

Government spending on science and technology 1993



it will have to reconcile itself to spending more on higher education and on the direct support of research.

That does not diminish the importance of what has been achieved in the past few decades. Since 1960, for example, there has been a 40-fold increase of the numbers of students registered for degree courses (not including teacher training). In the last academic year, there were more than 1.1 million students altogether.

That is a substantial springboard for the evolution of the skilled cadres Mexico knows it needs. But the higher education system will have to grow by a further factor of at least two before the proportions of young people in Mexico going on to higher education are comparable with those in Britain, let alone France, the rest of North America and Japan. No harm if that cost is pencilled in even at this early stage. The extra cost of easing the financial deprivation of beginning academics is not easily calculated, but should not be zero.

On the direct support of research, the rate at which funds are now spent is limited by the number of good ideas in the heads of able people. If projects such as the repatriation programme (see page 793) succeed, the demand can only increase. The government should be prepared to satisfy it.

In reality, the past decade has been richer in rhetoric than performance. Total public spending on research as a proportion of GDP actually fell during the 1980s to 0.32 per cent, partly at least because of the routine pressures on the public budget. Some say that the government should set itself a target for the proportion of GDP to be spent on research and then stick to it, but that is not a sensible way of

## Organizations decoded

**CICESE:** Centro de Investigación Científica y de Educación Superior de Ensenada

**CIMAT:** Centro de Investigación en Matemáticas

**CINVESTAV:** Centro de Investigación y de Estudios Avanzados

**CONABIO:** Comisión Nacional para el Conocimiento y Uso de la Biodiversidad

**CONACYT:** Consejo Nacional de Ciencia y Tecnología

**IIIE:** Instituto de Investigaciones Eléctricas

**IMP:** Instituto Mexicano del Petróleo

**INAOE:** Instituto Nacional de Astrofísica, Óptica y Electrónica

**INSP:** Instituto Nacional de Salud Pública

**IPN:** Instituto Politécnico Nacional

**ITESM:** Instituto Tecnológico y de Estudios Superiores de Monterrey

**N\$:** new pesos

**PRI:** Partido Revolucionario Institucional

**SEDESOL:** Secretaría de Desarrollo Social

**SEP:** Secretaría de Educación Pública

**SNI:** Sistema Nacional de Investigadores

**SPP:** Secretaría de Programación y Presupuesto

**UAM:** Universidad Autónoma Metropolitana

**UANL:** Universidad Autónoma de Nuevo León

**UNAM:** Universidad Nacional Autónoma de México



administering research.

What is needed, rather, is an undertaking that the government will allow its spending to increase as long as there are worthwhile projects to back. If that means a doubling of the present proportion of GDP over the next two decades or so, the government should count that as success.

**Industrial research.** Despite the way in which successful Mexican companies at Monterrey (see page 803) have ventured directly into research and development, and with the exception of state-owned organizations such as PEMEX (the oil company) and the electricity generating and distribution industry (which supports the Instituto de Investigaciones Eléctricas, a research and training institute at Cuernavaca), industrial research in Mexico is only meagre.

The government has taken sensible steps to provide infrastructure for the industrial research — US\$ 30 million of the World Bank loan will finance a National Metrology (standards) Centre, for example, while CONACYT is a partner in a kind of venture capital fund that provided funds of N\$81 million in 1993 for a variety of technical projects. There is also a CONACYT scheme for supporting joint research between industrial companies and academic laboratories similar in many ways to a scheme pioneered in the United Kingdom, as well as a number of government-supported centres aimed at improving the technical expertise of particular industrial sectors (for example, the shoe industry in León).

But at this stage in its development, and with NAFTA breathing down the collective neck, Mexico also needs a way in which companies that might benefit from R&D have an incentive to get their feet wet. Tax rebates for research are notoriously inefficient; some of what is called research is not research at all, some real research is not germane to the interests of the companies that take it on. But for a short time, say a decade, a tax-incentive scheme in favour of industrial R&D would powerfully test the seriousness of companies that might benefit.

**Morale.** The research community in Mexico is in reasonably good spirits; good luck for the government's ambitions for science and technology. The World Bank loan, coming on top of the earlier loan from the IDB, offers the promise of continued improvement. But there are also discontents. The parlous support for beginning academics is one. The general lack of personal mobility is another. But the most serious worry is that what is now accepted government policy will not be accepted tomorrow. That, of course, is why the assassination of Colosio, yet another sign that the future is unpredictable in a characteristically Mexican way, casts a shadow over science as well as the election. □

## A scientist's history of Mexico

Science began in Mexico with biological diversity. Mexican agriculture goes back 10,000 years. The chilis, beans and maize domesticated then are still staple crops today.

Civilization appeared about 1200 BC with the Olmecs of the Gulf Coast, whose culture seems to have spawned all subsequent civilizations in central and southern Mexico, most of which knew about mathematics, astronomy and — from their built structures — civil engineering. The fact that prehispanic culture flourished despite the lack of ferrous metals, wheeled transport or draught animals is no less remarkable for being often stated.

Mexican applies, strictly, to the Valley of Mexico, into which the ancestors of the Aztecs migrated from the north in the fourteenth century. The last Aztec emperor, Cuauhtémoc (a popular hero today) surrendered to Spanish conqueror Hernán Cortés (equally a popular villain) in 1521, bringing 3,000 years of history to a close, but for a legacy of more than fifty indigenous languages.

For the next 300 years, Mexico City was the capital of a colonial empire which, at its peak, stretched from northern California to Panama. The Royal and Pontifical University, the precursor of the modern National University, was founded in 1548, but the 'Royal and Pontifical' attribution led to its demise between 1833 and the revolution of 1910.

The declaration of independence from Spain in 1810 heralded more than 60 years of strife, during which Mexico lost present-day California, New Mexico, Arizona and Texas — more than half its territory — to the United States (1848) and was briefly governed by peripatetic Hapsburg puppet emperor, Maximilian (1864–67).

Maximilian was ousted by the lawyer-turned-politician Benito Juárez, a Zapotec from Oaxaca, who made education mandatory. In 1871 Juárez was in turn toppled by a colleague and fellow Oaxacan, Porfirio Díaz. Always an original, he died not by the bullet, but in his office, from heart failure.

The Porfiriato, as the 39-year dictatorship of Díaz and his associates came to be known, was a period in which democratic ideals were sacrificed for political stability and economic growth.

The revolution of 1910 ushered in a further decade of strife. The name of one of the revolutionaries, that of Cuernavaca's now-favourite son Emiliano Zapata,

whose tough peasant army effectively ruled Morelos and adjacent areas until his assassination in 1919, is that taken by this year's rebels in the southern state of Chiapas.

Order was restored to Mexico only around 1920. In 1929, Plutarco Elías Calles (president 1924–28) organized what is now the Partido Revolucionario Institucional (PRI). A successor, Lázaro Cárdenas (1934–40) confiscated private oil interests and formed Petróleos Mexicanos (PEMEX), still the national oil company. He also began the tradition of nominating his successor — invariably elected president — from the ranks of the PRI.

Curiously, the public administration of Mexico has survived these upheavals. The 31 states of Mexico, are in principle autonomous (and run their own universities, for example). Each has its own governor, who is formally elected.

Mexico's enforced self-sufficiency during World War II led to a postwar economic boom, during which Miguel Alemán Valdez (1946–52) built the modern campus of the National Autonomous University of Mexico (UNAM). A decade later, UNAM students were in the thick of protests against the administration of Gustavo Díaz Ordaz (1964–70); hundreds died in clashes during the 1968 Olympics in Mexico City.

Mexico's stock as an oil-producing nation rose during the OPEC oil embargo of the early 1980s, when the government borrowed heavily against future production. Then the bubble burst with the oil glut of 1982 and Mexico was plunged into a debt crisis. The gloom was accentuated by the Mexico City earthquake of 1985, when more than 8,000 died.

There has been an organized political opposition in Mexico only since 1988, when Cuauhtémoc Cárdenas (son of the former president) left the PRI to form the centre-left PRD (Democratic Revolutionary Party) in time for the 1988 election. But PRI candidate Carlos Salinas de Gortari won the election amid widespread allegations of vote-rigging, but with the smallest margin in history (50.36 per cent). The effectiveness of the political reforms he has instituted may be apparent in this year's election, due on 21 August.

Salinas's economic reforms are more palpable. Between 1988 and 1991, inflation fell from 52 per cent to 18.8 per cent: suddenly Mexico was the place to invest. NAFTA will further speed economic development. But social problems are as pressing as ever. The Zapatista rebellion in the rural southern state of Chiapas has been ended by negotiation, but its underlying causes remain. □



Olmec beginnings

# Bridgehead of science reform

Fausto Alzati could be running for election as President of Mexico one day — he has the presence — but meanwhile he is director-general of the Consejo Nacional de Ciencia y Tecnología (CONACYT), the chief conduit for research funds whose influence on Mexico's research is proportionately even greater. His pride is the reorganization of what used to be a bureaucratic organization — the administrative staff is a third of what it was five years ago.

But Alzati's real claim on public attention is to have made CONACYT transparent. Applications for research funds must be submitted formally, are sent out for peer-review and adjudicated by committees including members from overseas. Similar procedures are followed in the council's other distributive function — bursaries for PhD students, for example.

Jointly and severally, the research community seems to agree that the reform, a central feature of the government policy on science and technology implemented in 1992, is actually working well. Alzati seems to have attained his goal of winning the trust of the research community. The problem now is to keep that reputation for plain dealing.

In one intriguing way, the World Bank loan will help to keep the council on the path to probity. Washington's decision to make the loan was influenced to a large degree by the reform at CONACYT; now the council's programme officers are able to explain to client researchers that apparently impertinent questions (What has happened to that mass-spectrometer we paid for?, for example) are made necessary by CONACYT's international obligations.

CONACYT's influence on research in Mexico hangs partly on the diversity of its support programmes; if you want a spectrophotometer or a bursary to send a student overseas, CONACYT will be its source. But there is also a constitutional requirement (since 1970) that CONACYT should be consulted on all aspects of government policy affecting science. Although there is now a President's Council on Science and Technology as well, CONACYT is a prime mover in its own right. CONACYT, for example, has made the running in the operation of the system for accrediting active researchers under the label of the Sistema Nacional de Investigadores (SNI). The origin of the scheme, a decade ago, was to find a way of supplementing the salaries of able people during the then financial crisis. It has become a way of paying people extra if they are active in research.

The benefits of membership are considerable. At the top of the scale (Level III), a researcher will be paid a tax-free

salary supplement of N\$3,870 a month (about US \$1,100). Even for senior people, university salaries may be less. (UNAM pays just over N\$5,000 a month in salary to tenured professors, to which up to N\$3,000 of incentive payment may be added.) People on levels I and II earn respectively 60 per cent and 70 per cent of the SNI payment at level III. Those in the lowest order, that of Candidate, earn an SNI supplement of 40 per cent of that at level III (which, interestingly, is defined as 10 times the national minimum wage).

In reality, then, CONACYT determines not merely the distribution of research funds, but to a large degree the salaries of those who use those funds. Will it appreciate the need to deal differently (than at present) with people at the beginning of careers as researchers?

In the event, the SNI scheme has also become the most commonly used index of the well-being of a research institutions: how many SNI people does it have, at what levels? (One parameter in the CONACYT formula for deciding a research laboratory's annual income is essentially the average SNI supplement for the laboratory members.)

The total SNI membership, for example (at 6,600 odd) is a measure of the active research force. But 40 per cent of these are Candidates, and may never qualify at a higher level. For Mexico as a whole, only 5 per cent of the SNI force is at level III. Given that the definition of candidature can be extended to include those who have yet to obtain their PhDs; that just 170 people in the entire country obtained their PhD last year; and that even the small proportion of GDP spent on science is greater per scientist than that in the United Kingdom — then the number of scientists in Mexico is small indeed. This explains the urgency behind CONACYT's repatriation programmes (see opposite).

Like every other government appointee, Alzati and his team are due to hand in their keys when the new administration takes over on 1 December. CONACYT's influential science director, Dr Miguel Yacamán — largely seen as one of the architects of CONACYT's transparency — will go back to theoretical physics. Will the sweeping changes they have instituted be swept away in their turn, or will the next President keep up the pace?

Even with the assassination of the PRI presidential candidate, Mr Colosio, the feeling is that the change will continue. Recent history may provide a clue to the outcome.

Before he came to CONACYT in 1991, and during the negotiations to secure the loan from the World Bank, Alzati was an official at the Secretaría de Programación

## Homeward bound

**Mexico City.** The lack of trained researchers is the most pressing problem in Mexican science. Hence two CONACYT initiatives to boost research numbers.

The first aims to recruit people from outside the country. About 350 foreign scientists now live and work here on a permanent basis. Many come from Eastern Europe and the former Soviet Union.

The second is to exploit the 'taco nostalgia' felt by the 3,000 Mexicans working for their PhDs abroad. Many of these will be unable to get a research job in the country in which they graduated, so a return to a ready-made and funded post in Mexico has its attractions. About 600 people have benefited from this scheme. As big fish in a still-small pond, many returning Mexicans feel that they have far greater opportunities for advancement in Mexico than, say, in the United States or Europe.

But for some Mexicans the recent economic upturn has come too late. Alfonso Mondragón graduated from UNAM and went to do his PhD at the MRC Molecular Biology Unit in Cambridge, UK. That was in 1981, when Mexico basked in its oil revenues and grants were generous. The subsequent slump left Mondragón and many of his contemporaries faced with no choice but to stay away.

Instead of going home, he did a post-doc in Harvard and is now an assistant professor at Northwestern, where he is working on the structure of the enzyme topoisomerase I (see *Nature* 367, 138-146; 1994). Eight years after the slump, he feels that he has put down too many roots to make a return to Mexico a viable proposition.

Another expatriate Mexican (who declined to be named) is deterred more by the practical and bureaucratic difficulties of doing science in Mexico, and plans to return only when he retires.

The irony is that it is people like Mondragón — up-and-coming researchers with a good track record — that Mexico needs the most. But only about half of Mondragón's expatriate generation have returned, a symptom, says Mondragón, of bad timing.

y Presupuesto (the Office of Planning and Budget, SPP). His boss went on to become the Secretary of Education, and, in due course, signed the agreement with the World Bank. The Secretary in question is Mr Ernesto Zedillo, the new PRI Presidential candidate. Should Zedillo be elected President, he will have a strong personal stake in the future well-being of science in Mexico. □



# UNAM: cradle of Mexican science

**Ciudad Universitaria, México City.** The Universidad Nacional Autónoma de México (UNAM) is not just an institute of higher education. It is also the national natural history collection, the national library, the national research centre and a vast high-school system involving about 350,000 people. With a quarter of the population of Mexico living in Mexico City, UNAM reflects Mexico's centralism.

The responsibility of prominence, and the likelihood of being copied, makes UNAM people uneasy. There have been centrifugal stirrings for some years (see opposite). There is also chafing at the red tape generated by the huge bureaucracy required to navigate each day. Yet UNAM was the cradle of science in Mexico. Most Mexican scientists still win their first degrees there, even if they go abroad for higher degrees.

Degree-level teaching at UNAM proper falls to the faculties, which confer degrees, but research is almost exclusively carried out at distinct research institutes. Growth keeps the faculties on the move. Thus the faculty of science (for historical reasons, distinct from the faculty of chemistry) has had to move twice since its foundation, in 1939, in an old colonial downtown palace: first into a high-rise

block in then-new University City in 1952, and second, in 1977, onto a mini-campus of its own. That is now a crowded antheap of 5,000 BSc and 800 graduate students. It would be worse if numbers of science students had grown as quickly as in, say, business and finance, where job prospects are thought to be better. (Even so, around 1,500 BSc students in the science faculty are reading for degrees in actuarial mathematics.)

But this will soon change, thanks to the Inter-American Development Bank (IDB). UNAM will as a consequence of the recent 10-year loan have an extra US\$230.5 million of capital to spend, half from its own resources (which means the government). The faculty of science is to move for a third time into a new US\$20-million building: half this sum will buy new equipment ranging from a transgenic facility to a planetarium. The aim, says faculty director, Rafael Pérez Pascual, is to change completely the way science is taught. UNAM has already begun building a new science library and auditorium complex.

A side benefit will be a properly constituted graduate school, housed in the vacated science block. Graduates will, at least, have somewhere to hang their hats. Whether the new arrangements will rec-

oncile the competing influences of the faculties and the institutes, and make graduate education more effective, remains to be seen.

The road to a research degree from UNAM may not be narrow, but it is long. BSc students who survive the freshman year still have three and a half to go, and must then produce a thesis that may take a further year. That is often the last straw; many students may then be too caught up in jobs and family commitments to fit in a research project. And although tuition is free, living in Mexico City is not, forcing a third of students to take jobs to pay their way. So there are two classes of students: fresh and eager morning students, and afternoon students who arrive after a full day's work.

Graduate students suffer much the same privation. With a stipend of around US\$500 a month, most take a paying job to make ends meet. Inevitably, that prolongs the agony: the average length of a PhD course is as much as 12 years. And such is the shortage of fully qualified staff that dogged part-time graduate students are often members of the faculty themselves, teaching BSc students as well as one another.

The alternative, followed by many, is to study for a PhD abroad, encouraged by a

## UNAM playing the numbers game

UNAM's overwhelming problem is its own rate of growth. Take the high-schools. There are now 14 UNAM schools, with a total of around 120,000 students for whom entry to UNAM proper is all but automatic and almost independent of academic attainment — and about half of UNAM entrants are from UNAM schools.

Thus have the good intentions been turned upside-down: a system designed to prepare young people for the world's largest university produces one of the world's biggest dropout rates, at least in the first year of the BSc degree.

The problem is largely related to salary. If a graduate student gets N\$1600 (US\$500) a month, a new faculty member returning from a postdoctoral overseas might earn N\$2,800 (US\$875). The same person ten years on would command N\$3,500 (US\$1,100), and a full professor would earn around N\$4,000 (US\$1,250) per month.

These figures may be matched by internal UNAM productivity bonuses as well as membership of SNI (see page 791) the importance of which increases with seniority: a senior researcher with a string of publications in reputable

journals could earn N\$12,000 (US\$3,750) per month. But although these extras are taxable, they do not count towards superannuation, so it is hard to judge what a person's gross salary really means in terms of lifetime prospects.

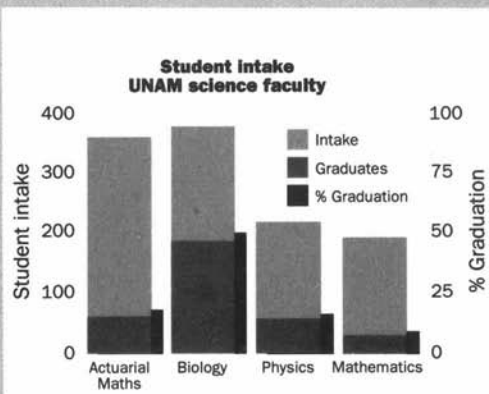
These cavils aside, if a senior postdoctoral in the United States earns as much as a professor in Mexico, the incentive is to stay away, even if the standard of

living in Mexico is competitive and the opportunities for advancement greater.

If even senior university teachers are badly paid, high-school teachers will always be poor relations in salary and status. Recruiting better people will not be easy. But more demanding requirements for entry to degree courses would be provocative — witness the climb-down a few years ago after noisy demonstrations against a proposal to levy a token tuition fee.

Another problem for UNAM — and for UAM, too — is that academics are government employees, so their prospects for advancement are closely tied to those of clerical and domestic staff. In April, it was not possible for outsiders to visit the campus at UAM because of a general strike over just such questions of status.

Giving UNAM high-schools more autonomy in the hope of shaking them loose at some later stage is the preferred solution of academics, but Mexican officials prefer to equate centralization with control — and call it accountability.



The intake of freshmen in the UNAM faculty of science, compared with the output of BSc graduates. Figures are for 1993: data courtesy UNAM.



Science faculty director Rafael Pérez Pascual guides visitors around what will be a library and auditorium complex.

system of bursaries that is generous towards expatriates (a cause for resentment among those seeking to fill their graduate programmes). Expatriate PhDs overcome by 'taco nostalgia' return to galvanize research at home. But there were never enough to establish a consistent tradition,

and the oil crisis of the 1980s led to a brain drain and deterred expatriates from returning.

To make things worse, the wage structure for newly qualified researchers is an encouragement to change careers at that point — taxi drivers and plumbers earn

more. The depopulation of science is being countered, actively, by the repatriation programme of CONACYT and the performance-related bonus scheme established by the National System of Researchers (SNI) (see separate article), but the process will be slow. UNAM helps it along by providing its own bonus scheme, and other universities and institutions do the same.

After a few more hard years, young Mexican scientists can achieve positions of seniority, even if only on the stipend earned elsewhere by a postdoctoral researcher. And this is another cause for concern — the lack of a class of professional scientist means that the few that there are tend to be railroaded into taking on administrative duties while still in their prime as researchers. Postdoctoral positions are almost unheard of, so there is little opportunity for the slow accumulation of technical expertise.

These are the palpable wellsprings of Mexico's dire shortage of indigenously qualified researchers. The problem and its origins are widely appreciated. But while only those graduate students with private resources (or an obsessive compulsion for science) are likely to succeed, improvement will be slow. □

## Resisting the traditions of the centre

**Ciudad Universitaria, México City.** Is UNAM too big? And should it be broken into smaller more manageable parts? These questions are rarely put in such blunt terms. Shame at the concentration of most things Mexican within the Federal District is only part of the reason why universities and research organizations have for twenty years nursed centrifugal tendencies. There are also benefits of amenity and cost. And divestment from the centre is government policy.

UNAM has been a leader in the field, using its research institutes to pollinate campuses elsewhere. The Institute of Physics, for example, now has branches in Ensenada and Cuernavaca, with interactions on the ground with students and staff from the local universities.

Local reaction to the presence of UNAM varies from resentment to enthusiasm that UNAM is addressing an unfulfilled need: because the university can fill its places from local intake, UNAM itself has a tendency to admit students from the regions only if they are unable to study their intended subjects locally.

To some extent this explains why UNAM is keen to set up branches outside Mexico City: it's a case of Mohammed having to go to the mountain. Although the faculties will be the main beneficiaries of the IDB loan, the Institutes will also get their cut: the institute of Physics, for example, is using its US\$6 million to build

and equip new facilities in Cuernavaca and Ensenada.

Ultimately, this diffusion could break the self-consciously monolithic UNAM and produce a much more loosely organized federation, (similar to the University of California, perhaps). Even the rector has voiced this idea out loud, and it's now a live issue.

Few other institutions have the maturity and critical mass to do this safely. One of them is CINVESTAV, the research wing of the Instituto Politécnico Nacional, with centres in Mexico City, Mérida (in the Yucatan), Saltillo (Coahuila) and Irapuato (Guanajuato). Without having obliged to take students of variable quality from its own high schools, CINVESTAV has managed to maintain standards by taking its pick of the best students in the country. Its success has also been achieved by a powerful sense of purpose. The Irapuato unit is concerned, exclusively, with plant genetics. At Saltillo, on the other hand, they are strong in materials and ceramics (and participated in the ill-fated Superconducting Super Collider across the border).

Twenty years ago, UNAM's bureaucracy and inertia inspired Mexico's National Association of Universities to try an experiment. Back in the 1970s, academics had the chance to create a brand-new university that would be free from the burdens that Mexican society has long

obliged UNAM to bear. The result was the Universidad Autónoma Metropolitana (UAM). To get away from UNAM's divisions into institutes and faculties, all UAM's teachers (four-fifths of whom are full-time employees) participate in teaching and research.

Again unlike UNAM, UAM was decentralized from the beginning, with three campuses in far-flung corners of Mexico City. Each campus has acquired its own character, but one thing remains the same: no campus is allowed to have more than 15,000 students. All three are filled to capacity, but a fourth campus is not an option at present. This has forced UAM to raise its admission standards (a move that would cause an outcry at UNAM) and pay more attention to its (as yet) minuscule graduate programme.

UAM is as yet too young — and has too few researchers — to start setting up branches in other parts of the country. Given UAM's credo it is tempting to speculate on how these branches will turn out. Will they be specialist research units, capitalizing on the strengths of the present campuses (biology from Xochimilco, engineering from Azcapotzalco), or will they be whole new campuses, along the lines of the first three, but with their own distinctive identities? If the future of Mexican universities is federal, might not the palm go to UAM, rather than UNAM? □



# State universities — building bridges

**Cuernavaca.** On paper, at least, Mexico is a federation of states. And despite the concentration of political and administrative power (and a quarter of the country's population) in Mexico City, each state outside the Federal District retains one significant talisman of autonomy: a university system of its own, similar to the state university systems in the United States. There is also a modicum of research.

The state universities huddle in the shadow of UNAM and the metropolitan universities, even in the eyes of many who teach in them. One reason is that many are poorly supported, another is that able academics cannot be prized away from the national capital. But the chief reason is that the state universities do not enjoy the constitutional autonomy of which the metropolitan universities boast. Instead, their decisions often depend on the political and personal relationships between university administrators and the state government.

The federal government would like to change all that, for budgetary reasons and because the state universities are potentially a more prolific source of enhanced skill. Yet it is rare to find academics who have moved from, say, UNAM to the payroll of a state university. The difficulty of mounting long-term research programmes is one deterrent; facing the resentment traditionally felt towards city-bred *chilangos* is another.

So there is emerging a mechanism of diffusion by the siting of central research institutes (or branches of them) alongside state campuses, without eroding their



The mathematics institute CIMAT, in Guanajuato, and its director Angel Canavati: "Escher is our favourite architect."

autonomy. Shielded in their protective bubble, rusticated researchers can pursue research as they would in Mexico City, while teaching courses at the university; then, when things go wrong, they can batten down the hatches.

Several central institutions now follow this strategy. In addition to UNAM's research institutes, the 30 research institutes now governed by CONACYT are similarly sited, as are the institutes of CINVESTAV, the research arm of the Instituto Politécnico Nacional (National Polytechnic Institute). Even ITESM, the well-regarded private technical institute of Monterrey (see page 803) has 26 campuses

in cities all over Mexico, often in association with local industry.

Like travellers in a strange place, research institutes seem to flock together even when their allegiance is to different central organizations. Around the University of Morelos at Cuernavaca, for example, there are two UNAM institutes (physics and biotechnology), two government laboratories — the Instituto de Investigaciones Eléctricas (IIE) and the Instituto Nacional de Salud Pública (INSP) — and an ITESM campus. And the University of Baja California at Ensenada shares its campus with two UNAM institutes (physics and astro-

## Making things add up at state level

**Guanajuato.** Among state universities, the University of Guanajuato is well favoured. Indeed, it basks in a political climate that favours research. The state governor is an enthusiast (and one of the three in Mexico who do not belong to the government party, the PRI). There are five main campuses, each with a slant towards a particular field. The Guanajuato campus itself is heir to the city's heritage as a mining town. Mexico was once the world's biggest producer of silver, and most of it came from here: the surrounding countryside is riddled with old mine workings.

Necessity, the mother of invention, has made the city's abundant supply of mining spoil-heaps the inspiration of the university's research interest in the extraction chemistry of mining residues. This has led to promising research into bioinorganic chemistry (especially silane halides), chemical engineering and theo-

retical inorganic chemistry.

But research here (as elsewhere in Mexico) is very young, and is hampered by cramped quarters and outdated over-used equipment, making even the most routine projects appear heroic. Researchers at UNAM and CONACYT institutes, more likely to have worked abroad, have a finer appreciation of their equipment needs and have the resources to tackle them. That is one reason why people at state universities set great store by alliances elsewhere.

But the University of Guanajuato is one among state universities at which the government's policy of spinning off specialized units from the Federal District seems to be working well. The Irapuato campus of the university, for example, boasts of its agronomy, at least in part because of the presence of the CINVESTAV plant biotechnology facility nearby (see opposite).

The university is also strong in pure and applied mathematics, thanks to the presence of a ready-made faculty from the Centro de Investigación en Matemáticas (CIMAT), the SEP-CONACYT mathematics institute up the hill, which is in itself a remarkable sight (above), perched 150 metres above a cavern large enough to house the cathedral in Mexico City.

When CIMAT began in 1980, its goals were in pure mathematics, purely. But reality has crept in. The nearby city of León, Mexico's chief centre for leatherwork, especially boots and shoes, has embarked on automation in the shoe industry to meet increased foreign competition. And CIMAT's geometers and algebraists have been called in to work on computer-aided design packages that could speed up the previously leisurely process of designing shoes to confirm with changing fashions.



nomy) and a SEP-CONACYT institute (CICESE).

There is no sign that this diffusion will suddenly stop, either: UNAM says it is always on the lookout for state university campuses willing to play host. It is about to send 20 neurobiologists to the city of Querétaro to found an institute which, with luck, will have close ties with the eponymous state university.

Moving an institute into the country physically is the easy part: building intellectual bridges can be more difficult. That is what the UNAM Institute of Physics found in 1983, when it sent a research group across the mountains to set up a branch next to the University of Morelos, in Cuernavaca. The objective of links with the University of Morelos did not materialize as quickly as had been hoped. UNAM researchers say Morelos's physicists welcomed their arrival, but social scientists and physicians were suspicious.

For their part, relocated UNAM scientists think that state universities need all the help they can get. Some say that if the state universities are to continue to call themselves universities they should improve or close down or describe themselves by some less elevated term.

Academic snobbery? Yes, to a degree. But there is also a practical problem for Morelos: without a pure science faculty, the university cannot supply the institute with students or faculty able to benefit from what it has to offer. So, belatedly a science faculty is being built with help from UNAM staff.

Nevertheless, the future will see the institute as the catalyst for the emergence of science teaching at Morelos. Three years ago there were three SNI researchers at the University of Morelos: now there are 30. Eleven years after its foundation the UNAM institute has around 20 faculty and 58 postgraduates (around 10 doing PhDs). The house speciality is molecular and atomic physics using low-energy particle accelerators. The institute's success has depended, in part, on personal contacts (between the rector of the University and Guillermo Soberón, then the rector of UNAM) and on a degree of political commitment that transcends the usual six-year election cycle. □

## The Irapuato approach

**Irapuato.** All Mexican laboratories have one objective in common: to attain what they call 'critical mass.' For everybody subscribes to the view that nowhere is there of a body of qualified people sufficient to foster and maintain a tradition of high-quality research, if necessary through political ups and downs.

Ariel Alvarez, director of the CINVESTAV Plant Biotechnology Unit at Irapuato, has a very clear idea of how that should be done. He sees Irapuato as a 'factory' for turning out PhD-level researchers in plant biotechnology, who can then be transfected to state universities to form inocula of excellence. But how to avoid the usual pitfalls of working with state universities?

First, be single-minded. Contrary to the Mexican norm, Alvarez has no real interest in turning out MScs with expertise in applied science; plenty of other institutes do that already. Irapuato's goal is to take in people with fresh BSc degrees and to turn them into doctoral candidates. One product is a greater number of younger people with PhDs. There is, of course, a research programme as well.

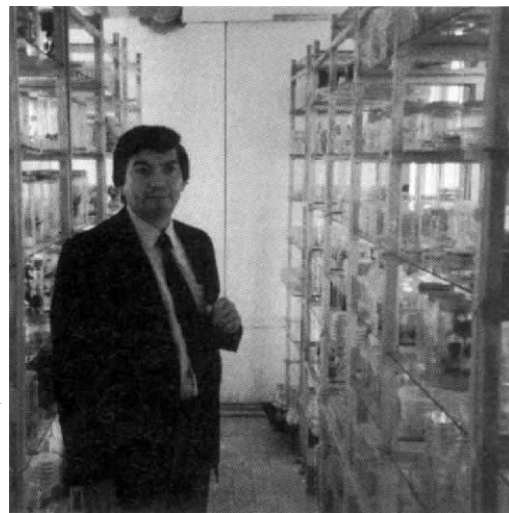
So Irapuato's MSc programme, begun in 1983, is already being phased out. Of the 66 graduate students now at the laboratory, 55 are registered for PhDs, roughly the inverse of the ratio at most other Mexican institutes. In the same spirit, 28 of the 32 faculty now have PhDs — again, an unusually high proportion for Mexico.

But that does not mean that new-minted CINVESTAV PhDs slip comfortably into ready-made posts at Irapuato. Instead, they are exhorted to go abroad for postdoctoral experience (postdoctoral positions being almost unknown in Mexico), and return only if they have 'something new' to offer the institute. Inbreeding, the usual trap when the science base is very small, is anathema.

And keeping a perch at Irapuato is tricky. Tenure has been replaced by renewable contracts and a system of eval-

uation that includes length of service and participation in international symposia as well as the usual publication record. Researchers are expected to bring in their own grants, which provide 75 per cent of the institute's income.

How does this help improve the lot of state universities? Irapuato's strategy is ingenious, and follows the Spanish colonial model of the fortified monastery. Rather than return a new PhD to his or her state university of origin, alone and unprotected, the unit 'packages' several new graduates together and uses them to minister high-quality science to state universities. The recipient university, for its part, agrees to leave the novitiate alone for two or three years to set up a laboratory unhindered by teaching or administrative duties.



Alvarez, back home, among the plant cultures.

In the end, everybody is happy. The state university gets its own graduates back, with interest. Irapuato fulfils its mission. And CONACYT, asked to outfit a single laboratory for three or four researchers rather than one for each, regards the strategy as a cost-effective way of tackling both the problem of critical mass and that of keeping trained people in Mexico. So far, Irapuato has despatched three of its missionaries to the University of Aguascalientes, three to Querétaro and two more to Sinaloa.

Irapuato seems to have overcome many of the practical problems faced by Mexican researchers. Luis Herrera was a pioneer in plant transfection technology while still a PhD student in Belgium (see *Nature* 303, 209–213; 1983) at about the time the Mexican government decided to add plant biotechnology to its traditional portfolio of agricultural research. So the Irapuato unit was given a spacious new building and encouraged to go head-hunting. Herrera and Alvarez were among the first to be repatriated. □

## Spreading the word

Self-interest implies that the policy of spreading education throughout Mexico is widely shared by public organizations; how else can modern skills be found in rural Mexico? Thus the Instituto Mexicano del Petróleo (IMP), a research laboratory in Mexico City supported largely by the national oil company PEMEX, is directly responsible for 30 education centres scattered among the oil company's facilities.

IMP boasts that 57,000 PEMEX employees attend these schools, whose timetables are arranged to suit shift-workers. The 5,400 courses on offer range from simple clerical skills to MSc courses in chemical engineering or computing (in conjunction with metropolitan universities). Most of the beneficiaries are in the oil-rich isthmus of Tehuantepec, where PEMEX recruits from unskilled peasantry.



# Ensenada, the Cinderella of the Pacific

**Ensenada.** This is the northern outpost of Mexico's research. This town sees itself as Cinderella of the Pacific, with the sprawling border cities of Tijuana and Mexicali playing the Ugly Sisters. Just above the seaside campus of the University of Baja California is CICESE (Centro de Investigación Científica y de Educación Superior de Ensenada) and two UNAM institutes. Each founded for its own reasons, they now form a strongly interdependent unitary campus.

Although the coastal location would suggest a taste for oceanography, research here began with the base camp for the National Observatory at San Pedro Mártir, 2,840 metres above sea level. CICESE itself was formed in 1973 as part of a government plan to decentralize research in Mexico. Starting salaries larger than those at UNAM were offered as an inducement to move out to what seemed like the ends of the Earth.

Twenty years on, CICESE is now the linchpin of research in Ensenada. The erstwhile telescope workshop is now the Division of Applied Physics, with interests in optics, electronics and telecommunications.

Given the San Andreas fault (which almost separates Baja California from the rest of Mexico) there is also seismology. CICESE's Division of Earth Sciences helped advise the Mexican civil engineers on how to strengthen buildings following the 1985 Mexico City earthquake. More

recently, the rich mineral deposits of the region (particularly copper), whose origins may reflect local tectonism, have become the focus of much interest.

Oceanology is the largest of the three divisions, concerned with marine biology as well as physical oceanography and climate studies, and the one with the strongest interaction with the University of Baja California. The centre's ecologists, until recently preoccupied with



Looking out: selsmography is important on the cusp of the San Andreas.

the oceans, are increasingly turning their attention inland to the fragile desert ecosystem of Baja California.

Ensenada, like Cuernavaca (see page 794), also boasts a branch of UNAM's Institute of Physics. In 1979, UNAM's

then-Rector, Guillermo Soberón, was bent on decentralizing science from Mexico. Ensenada was a strong candidate, given that the germ of the Astronomy Institute was there already, that CICESE, founded six years before, had established adequate infrastructure and that the university had started teaching undergraduate physics. In the event, UNAM sent to the north the materials science part of the solid-state physics effort.

The distance from Mexico City has also encouraged a diversity of students, not only from Baja California and Sonora but even from as far away as Yucatan. Higher degrees are awarded by CICESE, not UNAM.

Remoteness has also allowed the UNAM Institute to evolve in quite unexpected directions. Its workload ranges from catalysis and high- $T_c$  superconducting films to quantum chemistry and neural networks. It has become a

national facility for materials science and (it hopes) industrial research, unique in Mexico. It hopes that, with an injection of US\$1 million from the Inter-American Development Bank loan, it will soon have a new building. □

## Health matters in transition

**Cuernavaca.** The problems of public health in Mexico are those of a country with a rapidly growing and mobile population, in transition from southern poverty to northern prosperity.

In the torrid south, the problems are malnutrition, diarrhoea, parasitism and infectious diseases. But nobody has died from malaria in the past five years and Mexico may soon receive international certification for the eradication of poliomyelitis. Nevertheless, there are still isolated patches of onchocerciasis, dengue, cholera and Chagas disease.

Part of Mexico's answer, in 1987, was the foundation of the Institute of Public Health (Instituto Nacional de Salud Pública, INSP) in Cuernavaca, for teaching and research. This combines the old Escuela de Salud Pública de México (founded in 1922) with two research units, one specializing in infectious diseases (Centro de Investigaciones sobre Enfermedades Infecciosas, or CISEI).

From the outside, the INSP is one of the bolder and more startling creations of

modern Mexican architecture. Whether the activities inside are yet geared up to meet Mexico's problems is another matter. Chagas disease, the New World's version of African sleeping sickness, illustrates the difficulties. The most worrying feature of the disease is that the causative agent, *Trypanosoma cruzi*, is elusive. So although the disease is plainly evident in Oaxaca and other parts of southern Mexico, it does not show up in autopsies and blood for transfusion is not screened for it.

Yet in the mid-1980s, 3.8 million Mexicans may have been carriers (transmission is by bug *Triatoma mazzottii*) — a huge pool of latent infection. And yet, according to Raúl Ondarza, director of CISEI, precisely this latency may explain why the epidemiology of the disease is still obscure and why Chagas does not get the attention its seriousness deserves. Further north, diabetes, cancer and high blood pressure are increasingly the worries, but Mexico City's pollution, anathema to asthmatics, is something special

to Mexico. And then there is AIDS.

The current estimate is that the rate of infection with HIV is 30 per cent in homosexual men and bisexuals, but less than one per 1,000 among heterosexuals. The worry is that Mexican men contract the disease while working as migrants in the United States. So although Chagas falls through the net, blood is scrupulously screened for HIV.

Tuberculosis has increased with AIDS, but there has been no respite in the vaccination effort. Nineteen out of twenty Mexicans are vaccinated against tuberculosis, even in remote Chiapas.

INSP is still a young institution in the course of finding its feet, but that shows. Many of its laboratories have yet to acquire a 'lived-in' feeling, seeming pristine even by the standards of the still relatively new UNAM Institute of Biotechnology next door. And although some researchers badly need certain items of equipment, they are sometimes surrounded with other machinery surplus to requirements. □

# Against the grain

Arguments about land lie at the heart of Mexico's earlier revolution and are at the heart of the discontent that surfaced in Chiapas, Mexico's southernmost and poorest state, in January this year.

Mexico's peasant farmers have long been victims of economies of scale, as big growers force down prices of maize and coffee. The Chiapas uprising was no surprise to Eckhart Boege, an archaeologist turned anthropologist from the National Anthropology Institute, who has been studying Indian groups in Oaxaca.

But others, such as expatriate British historian David Skeritt from the University of Veracruz and also Xalapa sociologist Hipolito Rodriguez, were taken by surprise. They had expected that if discontent were to surface anywhere, it would be in the major cities.

The theory is that those who leave the land join the urban poor, and even Salinas' economic revival has not met the need for a million new jobs each year. But economic reform has created a further problem; inflation has been fought with cuts in real wages, making moonlighting a necessity for many people, especially the middle classes. Academics remark that the first wave of industrialization brought social divisions that helped to trigger the 1910 revolution.

Chiapas has shifted the focus to the development needs of the rural poor. What will the development of science do for them?

The pattern of Mexican science is attuned to the need. The Mexican view of the life sciences is through a glass stained by biological and cultural diversity. Biology means old-fashioned zoology and botany. Conservation includes wild spe-

cies and domesticated varieties equally. Genetics means agriculture.

Experimental biology is either called biotechnology or lumped with "chemistry" or "physics". All is influenced by anthropology. It is not for nothing that conservation legislation is enforced by SEDESOL, the Secretaría de Desarrollo Social (or the Ministry of Social Development). Thus although CINVESTAV's Irapuato laboratory (see page 797) is a thoroughly modern plant biochemistry and genetics laboratory in which any genetic engineer would feel at home, its researchers proclaim the importance of Mexico's cultural and gastronomic heritage.

So plant transfection pioneer Luis Herrera is working on transfection systems for traditional crops such as maize, beans and papaya. Mercedes López is busy teasing apart the biochemistry of *Ustilago maydis*, a fungal pathogen of maize, itself a gastronomic delicacy called *huitlacoche*.

There are more mundane problems to solve. Compared with bread wheat, for example, little is known about the nutritional quality of tortilla flour except that nutritionally poor zein comprises more than half the protein and is digestible only after the maize meal is soaked with lime before tortillas are made.

So Octavio Paredes from Irapuato is looking for ways of adapting the tortilla to the fast food culture without compromising its nutritional value. Others are seeking bean cultivars that will be more durable sources of the essential amino acids lysine and methionine, lacking in maize.

It is the "social tragedy" of Mexican food, says Paredes, that the culinary and horticultural traditions of subsistence agriculture now under threat are the very

## Green revolution

The green revolution of the 1960s began in Mexico. Its temple is the International Centre for the Improvement of Maize and Wheat (CIMMYT) at Chapingo, not far east of Mexico City. The centre was originally founded by the Rockefeller Foundation and now a founder member of the World Bank's consortium of international agricultural research centres.

Ironically, the green revolution may have made India famine-free — and the Indian Punjab prosperous — but it has done little for Mexico. The intensive agriculture that makes these strains of wheat flourish is not easily adapted to a country such as Mexico, where all but a small amount of farmland is on mountain slopes.

The emphasis at the Colegio de Postgraduados in nearby Texcoco has, therefore, shifted, and researchers are working on ways of improving farming methods in tune with Mexican needs.

But that has not prevented Abel Muñoz Orozco and his colleagues from the Colegio from scouring Mexico's myriad montane valleys for native varieties of maize (each valley has its own), testing them for qualities such as yield, resistance to disease and drought and — most importantly — their ability to tolerate the high-alumina volcanic soils of central Mexico.

same that have kept malnutrition at bay for centuries.

One solution for Mexico's tropical agriculture is to sustain several different activities on the same plot, leaving patches of managed forest to be exploited for fruits and wood — as coffee is traditionally grown. Boege and his colleagues are working with peasant farmers in different areas to find the appropriate mix of species and varieties in each case.

The key is being able to demonstrate immediate, direct benefits to people who choose to stay on the land. For example, Eugenia Olguin from the Institute of Ecology in Xalapa was involved in a scheme to encourage peasant women to grow water plants and use them as compost to foster earthworms. When fed to chickens, the resulting mixture produced — for the first time — a surplus of eggs which, when sold, allowed the women to buy meat for their families.

The Colegio de Postgraduados en Ciencias Agrícolas at Montecillo, near Texcoco, just to the east of Mexico City, is engaged in similar schemes. The researchers know they must demonstrate immediate benefits if their client peasants are to change their ways. But can they also demonstrate returns that will keep the big growers in check? □

## Fighting for tools of the trade

Fitting out a laboratory in Mexico needs patience. Even though CONACYT has reformed its grant allocation procedures, researchers still complain about the bureaucracy and delays that separate application and approval.

To make matters more difficult, the government levies a 60 per cent duty on equipment bought elsewhere. In theory, research equipment is zero-rated; in practice, customs officials may not know the difference between laboratory equipment and industrial plant, which might be liable for duty.

That has been a disincentive for US foundations, for example, who may wish to support science in Mexico, but not the Mexican exchequer.

NAFTA, which came into effect in January, should change things, al-

though slowly. Manufacturers from the United States and Canada will then have an edge over Europeans.

Meanwhile, researchers have learned to adopt guerrilla tactics. The expertise of commission-driven equipment salesmen in cutting through red tape is invaluable: for perishables, twice the price in seven days is better than waiting three months to pay the catalogue price.

These obstacles make it difficult to get projects up and running. But with no tradition of either postdoctoral research or of research grants lasting longer than three years, research groups with consistently high standards are few and far between. But there are some. No doubt there will be more.



# Astronomy: eyes firmly on the stars

**Tonantzintla, Puebla.** Modern astronomy in Mexico owes its character to one man, Guillermo Haro, co-discoverer of the protostellar nebulae known as Herbig-Haro objects. Thanks to Haro's influence, Mexican astronomy has ever since been drawn to the study of star-forming regions, molecular clouds, young stars and so on. Haro assumed the directorship of the national observatory in 1950, eight years after its move here from the bright lights of Mexico City, marked by its acquisition from Harvard of a fine 80-cm Schmidt camera.

That instrument has been the mainstay of research here ever since, but in 1971 Haro broadened the scope of the observatory, introducing studies cognate to astronomical observation. The result was the Instituto Nacional de Astrofísica, Óptica y Electrónica (INAOE), which became a CONACYT institute in 1992.

But the expansion of nearby Puebla led to another move for the national observatory, this time to the dark, dry and distant heights of San Pedro Mártir in Baja California. The observatory at San Pedro (now administered by UNAM) was started during the 1960s; it now has three telescopes of 80 cm, 1.5 and 2.1 metres.

What was once the 'base camp' for logistic support, on the coast in Ensenada, has blossomed into part of CICESE and a UNAM Institute. Not that this makes San Pedro seem closer: bereft of telephones and mains electricity, the observatory is supplied by trucks and diesel tankers which must negotiate a 100-km trip on metalled road — followed by 120 more on rough tracks.

There is now a prospect that a fourth telescope may come to San Pedro. The Carnegie Institution of Washington, pulling out of Palomar, is involved in a project to build the 'Magellan' 6.5-metre optical instrument at Las Campanas in



Old and new: the Sun temple at Teotihuacán and a modern telescope in Puebla.

Chile. With collaboration from UNAM and the University of Arizona, it is possible that Carnegie will underwrite a similar instrument at San Pedro. While they are waiting, UNAM astronomers are working with the University of Massachusetts on an infrared spectrograph for San Pedro using electronics developed in Mexico.

With San Pedro now in the UNAM orbit, INAOE itself administers the observatory at Cananea in the Sonora desert near the US border and 'within sight' of Mount Hopkins on the other side. A special interest in high-frequency electronics has led to INAOE's participation in a joint US-Mexican plan to build the world's largest millimetre-wavelength radio telescope.

The germ of this idea came from astronomers at the University of Massachusetts, who in addition to prior familiarity with San Pedro have had a 15-metre-diameter millimetre-wave dish and hopes of bigger things. Observations in the millimetre range require extremely

dry, still air, such as is found in the northwest of Mexico. Given that observations in this waveband are ideal for looking at just the sorts of things Mexican astronomers like to look at, the University of Massachusetts found ready partners at UNAM and at INAOE.

The largest instrument of this type at present is the Franco-German IRAM instrument in Spain, which has a diameter of 30 metres. The projected Mexican instrument will be 50 metres in diameter. The surface of the dish will be 'active', divided into 126 independently controllable hexagonal segments.

The cost is projected at US\$42.8 million, to be divided equally between Mexico and the United States (an equitable division to be reflected in allocations of user time, once the instrument is built). CONACYT is expected to put up half the Mexican half (a final decision is expected at the end of this month), the rest coming from other sources, hopefully from government of the state successful in luring the project onto its territory.

Perhaps for this reason, Mexican astronomers are coy about revealing the projected location for the instrument, although several sites have been evaluated. Those in the business of making educated guesses should know that apart from the necessary aridity, the telescope will have to be situated moderately far south to get a good view of the Galactic Centre.

On the US side, the state of Massachusetts is believed to have committed US\$5 million (provided that other funds are pledged), with the University of Massachusetts another US\$1 million. The Federal Advanced Research Projects Agency (ARPA) will add another US\$3 million, but the remaining funds have yet to be found. If all goes well, the telescope should start work at the turn of the century. □

## A predisposition to astronomy

Astronomy in Mexico was well-established when, in 650 AD Maya, Zapotec and Teotihuacán astronomer-priests met at the hilltop observatory of Xochicalco, Morelos, to synchronize their sundials. This Mesoamerican Calendrical Convention is arguably the first scientific conference on record — the poster session is still carved into the base of the pyramid.

The convention at Xochicalco could have been held to mark the coincidence of starts of the 365-day solar year or *xihuitl* and the 260-day ceremonial year or *tonalpohualli*, which occurred every 52 years. (The Maya

also had an absolute reckoning of days, similar to the Julian Day used by modern astronomers, using as a reference point 13 August 3113 BC.)

According to Jesús Galindo, an astronomer at INAOE, many temples and pyramids are positioned so as to mark the points on the horizon at which the Sun will rise or set on certain dates. The pyramid of the Sun at Teotihuacán is aligned towards the sunset on 29 April and 13 August. The first date is 52 days before the summer solstice, the second 52 days after; the rest of the year comes to 260 days, or one ceremonial year.

# Biodiversity: an unmapped resource

**Mexico City.** The humblest Mexican on the city Metro is proud of the country's biological diversity: how could it be otherwise, with all those street markets piled with fruits and vegetables not found elsewhere? But now Mexico has a biodiversity research programme as well as biodiversity.

With good reason. Although Mexico is the fourteenth largest country in the world, it comes first in the league table of reptiles (there are 717 of the 6,492 recorded reptile species in Mexico, 393 of which are found nowhere else) and second in respect of mammals (439 out of 4,300, of which 139 are endemic). Altogether, 10 per cent of the world's species can be found in Mexico.

These figures come from the Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO), a government agency with an office on a stretch of urban motorway in Mexico City, ironically next to a McDonald's restaurant and a motor showroom.

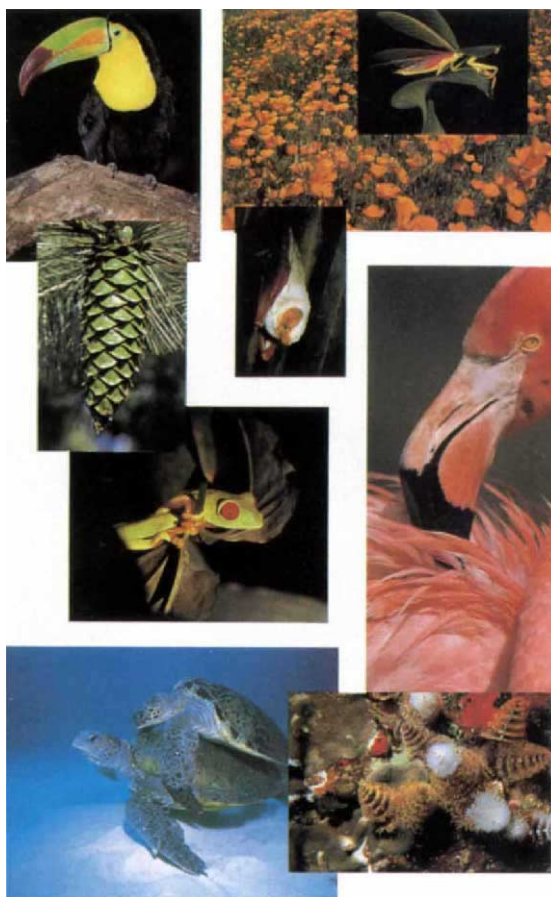
CONABIO is a ministerial commission set up in 1992 by presidential decree. Its status could hardly be higher. CONABIO's president is the President of Mexico, Carlos Salinas, and its committee comprises the rector of UNAM, the Secretary for Social Development and eight other government ministers.

Although formally founded in March 1992, it did not really get going until the autumn. So its foundation, at least, predates the 'Earth Summit' in Rio de Janeiro.

The aim is to promote knowledge and conservation of the Mexican biota, to which end it funds research projects and publicity ranging from taxonomic treatises to television programmes. The high-level central committee meets but once a year to decide the budget, which last year was US\$3.4 million, of which US\$2.4 million went to support projects.

Deciding which to support falls to the project evaluation director, Ana Luisa Guzmán, and her colleagues, who send likely applications out to peer review. Last year CONABIO received 208 project applications, of which 65 were approved. But CONABIO is surprisingly pro-active, commissioning 22 further projects in areas considered in need of support.

Mexican biodiversity is little explored, so CONABIO's priority is to produce taxonomic checklists and databases of specimens as quickly as possible. There have already been several books and publications. The latest initiative is the



Mexico's diversity -- pictured by CONABIO

establishment of a computer-based biodiversity information network, which Guzmán hopes will start in a small way this month.

Another plan is to store information about Mexican specimens held in museums outside the country, which has provoked suspicion that Mexicans are on the prowl for their own organic national heritage. Far from it, says Guzmán — Mexican museum space is already at a premium, but there is always room for one more entry on a CD-ROM.

Space is certainly tight in the zoological and botanical teaching collections of the UNAM faculty of science. Claustrophobes should seek work elsewhere. But the collections are due to move to what is now the faculty library, when that in turn is rehoused in larger quarters now being built. Even so, there will be no room for flora or fauna from outside Mexico unless they bear on problems of Mexican diversity. It may be fortunate that Mexico has few indigenous large mammals.

The national research collections are housed in the nearby UNAM Institute of Biology. Its mission for more than 60 years has been to augment and maintain them. In refreshing contrast with many countries in Europe or North America, there is a palpable sense among Mexican

taxonomists that there are still discoveries to be made, which keeps the museum disciplines alive and fresh. One result is that the Institute of Biology attracts students from all over the country. Another is that, despite the honour elsewhere accorded to molecular biology, to study biodiversity in Mexico is a matter of national pride.

Systematics may be the core, but CONABIO's remit is extraordinarily broad, encompassing ethnobotany and, with that, the anthropological dimension inescapable in a country with deep roots in agriculture, husbandry and indigenous lore. So a catalogue of, say, rare varieties of domesticated chili is as pertinent as a survey of butterflies and moths. But because most modern Mexicans have the genes of indigenes, the appreciation of biological diversity has profound cultural significance; that could explain the government's powerful backing of CONABIO.

One flagship project is to chart the flora and fauna of the coastal state of Veracruz, where 30 per cent of Mexico's vascular plant species are estimated to be found. At the sharp end is orchid specialist Victoria Sosa of the Institute of Ecology in Xalapa (see page 804). The checklist is now complete: on their way are the keys and descriptions, arranged by family. Volume 80 of this, the *Flora Veracruzana*, has just appeared — and marks the half-way stage.

The work also has a time dimension, incorporating records going back a century, so as to assess the impact of humanity on the flora.

CONABIO's heavy ministerial membership makes its future after the presidential election on 21 August uncertain. Guzmán is confident that the political will to support CONABIO will survive. But there is an intriguing hedge against inconstancy in CONABIO's funding structure: rather than being paid directly from the government, funds are held in trust and administered by the national development bank, Nacional Financiera.

Originally, that was meant to buffer CONABIO against political caprice and excessive bureaucracy, but it also allows funds to be carried over from one financial year to the next, and to be augmented by private donations. Research organizations elsewhere may envy this Mexican device for flexibility. The fact that CONABIO can run on such a shoestring and still contemplate coming in under budget shows an artful thrift — and demonstrates that in alpha taxonomy, a little can go a long way. □



# The world's biggest urban experiment

**Mexico City.** Cortés' first sight of the Aztec capital Tenochtitlán in 1519 was from the high pass between the twin volcanoes of Popocatepetl and Iztaccíhuatl. Now one is more likely to see a thick, brown blanket of smog.

With an estimated 20 million (nobody is really sure), Mexico City may be the biggest city anywhere; it is also one of the fastestgrowing, putting public utilities under huge stress.

Getting about, for example, has become a herculean task. The extensive urban rail system is habitually so crowded that commuters are forced onto the roads. The city authorities have responded by banning cars from the streets according to the last digit of their licence plates. The well-to-do thwart the rule by simply buying extra cars.

The topography does not help. At a low latitude (19°N) and high altitude (2,240m) in a valley encircled by even higher mountains, the city is a recipe for an almost permanent atmospheric inversion layer. The 20 per cent deficit of oxygen compared with sea level is amply compensated for by ozone, hydrocarbons, aerosols and particulates.

Because petroleum is partly to blame, it is only fair that the Instituto Mexicano del Petróleo (IMP) has been saddled with a project to define workable solutions. This is the Mexico City Air Quality Initiative, a US \$10 million joint project with Los Alamos National Laboratory and several other bodies in Mexico, the United States and elsewhere.

The idea is to monitor traffic flow as well as ozone and hydrocarbon concentrations throughout the Valley of Mexico and to use the data to test various strategies for cleaning up the city.



A smoggy morning in Mexico City. The large building on the right is the new stock exchange.

A total of 38 packages of anti-pollution measures has been tested in detail. Ingredients include extending the urban rail network, reformulating fuels, restricting the use of cars of a certain age or make, collecting effluent from paint-shops and even changing the hours of daylight-saving time. Each has been evaluated for environmental benefits and costs, tested using computer simulations and given an economic rating. The seven-volume report waits in the government's in-tray and for the August election.

Nevertheless, the problems of Mexico City — and pressure from the US administration during the negotiation of NAFTA

— spurred the government towards tougher legislation and enforcement in environmental protection. Mexico's General Law of Equilibrium and Environmental Protection, passed in 1988, superseded legislation passed in the early 1970s, but largely ignored. Nowadays it is enforced by inspectors from the Secretaría de Desarrollo Social (Ministry of Social Development, SEDESOL), the equivalent of the Environmental Protection Agency in the United States.

Under the 1988 law, new or modified facilities require SEDESOL approval before they can begin; the subsequent monitoring of air and water quality and waste management is vigilant. SEDESOL can (and does) close offending installations, and impose fines and imprisonment on their operators.

Not surprisingly, environmental consultancy is now big business. At least 200 consultancies now have offices in Mexico. One of them is the Texas-based Environmental Resources Management (ERM), whose speciality is negotiating the regulatory minefield for US clients thinking of moving south.

Scott Koolik came to ERM's Mexico City office three months ago, to serve as a friendly English voice to prospective clients. A major concern of US companies, he says, is the past history of a prospective industrial site. In the United States, present users are held responsible for the effects of contamination left behind by others, and the cost can be ruinous. Little attention has hitherto been paid to such problems in Mexico, but observers expect this to change in the next few years. □



An experimental petrol station at the Instituto Mexicana del Petróleo, now in the forefront of research to control pollution.



# Industrialists facing the NAFTA facts

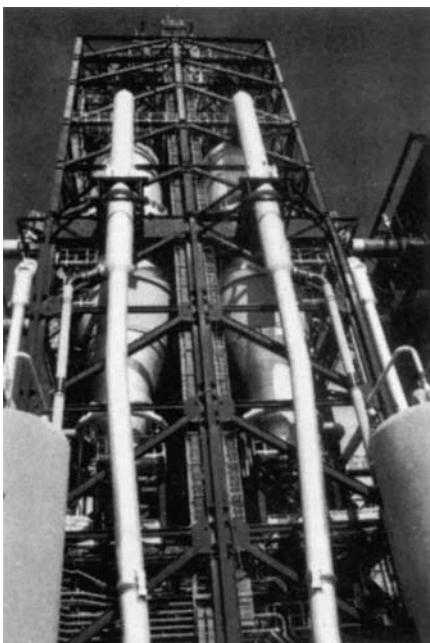
**Monterrey.** It all began with beer. In the 1890s a brewer called Schneider came to Monterrey to found the *Cervecería Cuauhtémoc*. But beer needs bottles and caps, so the brewers founded glass and steel works. The recent arrival of substantial banking interests has made Monterrey a thriving financial centre.

This vibrant atmosphere owes much to proximity to the United States. Monterrey people have long been used to cross-border commerce, so tend to be blasé about the changes that NAFTA will bring.

Nevertheless, there is a sense of expectation that, with NAFTA, companies from outside North America will take advantage of Monterrey's low costs and high technical expertise and locate their North American operations in the city. Mexicans might respond to NAFTA, ironically, not by heading north but by opening up markets in Europe. Conversely, an influx of Europeans and *Norteamericanos* could spur US universities to set up campuses here.

They would have tough indigenous competition. Education here has been pursued with vigorous single-mindedness, to supply private industry with qualified technical staff. Monterrey companies are large enough to invest in R&D (see box) as well as sponsor potential new recruits.

Many of these study at the public Universidad Autónoma de Nuevo León (UANL), Monterrey's biggest university, with 100,000 students. Many of the faculty come from industry – for example, every



The steel industry remains prominent.

seat on the PhD committee of the engineering faculty is occupied by an industrialist. But UANL is also strong in biotechnology and medicine.

A further 48,000 go to the private Instituto Tecnológico y de Estudios Superiores de Monterrey (ITESM). ITESM celebrated its golden jubilee last year and is unusual among private universities in its accent on science and technology. Three years ago, a conscious decision was taken to reinvent ITESM as a research univers-

ity. Since then the number of PhDs on the faculty has risen from 100 to 400, many from abroad.

Although based in Monterrey, ITESM has 26 campuses all over Mexico from Ciudad Juárez in the north to Chiapas in the south. Some classes are broadcast by satellite, and all campuses are interlinked by modem. Not surprisingly ITESM is strong in telecommunications.

ITESM's centre for sustainable development is housed in a brand new aquamarine glass skyscraper. In the basement are laboratories for research into air and water quality. The subjects become more inclusive as one rises: on the top floor is the Centro de Estudios Estratégicos, a social science unit whose task is to study the overall economic development of Mexico and its regions. Of special interest are studies on the economic integration between various border states and the United States.

The findings thus far suggest that NAFTA is a two-edged sword. Although it should create new jobs in Mexico, half a million of these will be immediately absorbed by a net movement off the land as agriculture becomes more intensive and more mechanized. At the same time, Mexican businesses will be forced to become more competitive, and many will fail. The result will be a net contraction in the number of jobs with Mexican firms in Mexico.

ITESM researchers have long seen the problem as one of economics and management as much as the development of tech-

## Closer to Houston than to Mexico City?

Monterrey will be one bellwether of the upheavals NAFTA brings. For this is where Mexico has most conspicuously set its sights on industrial competitiveness. The brewery's bottle-making operation, for example, has turned into a company called Vitro which, with 47,000 employees, claims to be the world's fourth largest glass-maker. And the foundry has grown into HYLSA, a steel-maker with a taste for innovation. For example, it is peddling a scheme for the direct reduction of iron ore by natural gas in plants which, it says, are small enough to be affordable in developing countries.

Piquantly, HYLSA's origin in the Cuauhtémoc-Moctezuma brewery is still apparent. HYLSA bottles waste carbon dioxide from its direct reduction process and sells it to Monterrey's breweries. Consider: the gas in your next Dos Equis (Cuauhtémoc-Moctezuma's best-known brand) may be natural.

These companies, and others, make Monterrey the industrial R&D capital of Mexico. Vitro began on R&D as long ago as 1978; now each of its divisions is required to spend at least 2 per cent of sales on R&D.

HYLSA is equally active. Since 1957, it has filed 945 worldwide patents. Apart from such things as the direct reduction process, it has protected a pneumatic bed technology for pumping semi-molten iron from the reactor direct to the forge. Monterrey companies are confident enough, and big enough, to take the patent process seriously.

Cross-border connections are conspicuous. People like to say they have more in common with Houston, Texas, than with Mexico City. Vitro is a proof of that: it sells US Corning products south of the border, while 30 per cent of its assets are in the US glassmaker Anchor. And HYLSA is a member of the American Iron and Steel Institute.

Monterrey also houses the man-made fibre makers CYDSA, the cement company CEMEX and the millers MASECA, Mexico's biggest manufacturer of tortillas, now aggressively exporting to South America as well as India.

The city's industrial base and technical skill makes it a magnet for industrial R&D. The Coahuila-based mining company Peñoles, one of the world's biggest producers of silver, has sited here its 40-strong division developing refractory blast-furnace linings, for example.

Commercial power arrived more recently, when Monterrey companies make a killing from the re-privatization of the banks nationalized in the 1970s.

So what will NAFTA do for (or to) Monterrey? One expectation is that companies from outside North America will relocate here to take advantage of low costs and high skill; Mercedes Benz is already building buses here.



## South of the border

**Tijuana.** Roadside verges south of Tijuana look as if they are where old cars go to die. Environmental damage in and around the 2,000-mile border with the United States is the responsibility of the *maquiladora* industry, which has brought almost 5 million people to the region.

The *maquiladora* programme was established in 1965 to attract labour-intensive industries to Mexico. The idea is simple — raw materials are imported from the United States duty-free and made into finished goods, which are then exported to the United States, together with all waste materials for disposal or recycling.

Eighty per cent of *maquiladoras* are strung along the border, the rest are found in Mexico City, Guadalajara and Monterrey. The plan worked: in 1965 there were 12 plants employing 3,000 people; now there are more than 2,000 plants staffed by close to half a million workers. They produce all kinds of things, but 44 per cent of production is related to electronics.

But economic success has brought environmental disaster. According to the Ministry of Social Development, SEDESOL, only 31 per cent of *maquiladoras* return their wastes to the United States as required by law — with only one permitted hazardous waste landfill in the country, the remaining 69 per cent gets into the soil and groundwater.

Population growth has outstripped infrastructure development. Belatedly, the government has set up industrial parks in an effort to provide better services for (and regulation of) the *maquiladoras*.

nical expertise. Business in Monterrey is conducted now much the same way as it has always been. Even though they are now powerful and profitable, Monterrey companies are still essentially family firms.

ITESM academics worry that this old-fashioned management style may prove a hindrance to Mexican firms exposed to the open competition promised by NAFTA. For more than a decade, ITESM has run what it calls the Centro de Calidad (Quality Centre), the aim of which is to develop modern management practices that offer high standards of service to clients, and yet retain a distinctive Mexican flavour. This kind of management consultancy, familiar enough in Europe or the United States, is less so to Mexican industrialists. But according to Maria Sanchez of the Centro de Calidad, nothing less than a cultural change will be needed to fit Mexico out for NAFTA. □

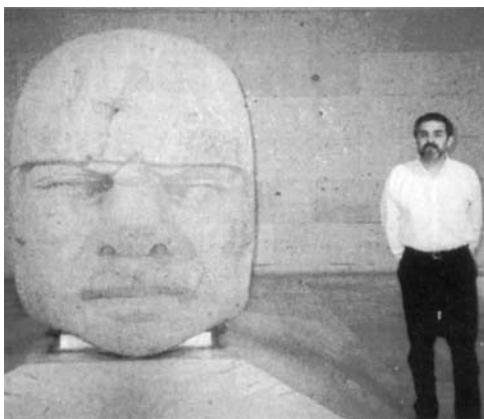
## More than just a film set

**Xalapa.** In the hills above Veracruz a month ago, Harrison Ford from Hollywood was making a film on an undisclosed theme in nearby Coatepec. A century before Paramount Pictures blocked the narrow streets with trucks, that town was the centre of the Xalapeño coffee-growing industry. Now the region has an ecological interest of its own.

The humid air rolling up from the lowlands condenses in the hillside forests, making a grey foggy climate like that of Wales, but warmer. The flora of the resulting cloud forest forest is unique; pines and planes rub along with cacti, bromeliads and Spanish moss.

Xalapa itself adds to the contradictions. It was once well-known for its chili market (whence Jalapeño), even though chilis do not grow here. And, far from the sea, it boasts a famous fish cuisine (cod with watercress sauce a speciality).

The rain forests that surround the town



Ecology Institute director Sergio Guevara (and friend).

are deceptive. Close up, they are coffee plantations gone to seed. Coffee used to be an environmentally friendly crop; many of the older varieties thrived on warmth but required shade. So coffee-growers added the bushes as ground cover within existing forests, exploiting the canopy's shade. Thus was produced a kind of semi-managed forest, to which other crop plants (such as bananas) could be added. This happy arrangement preserved and nourished the poor and acid soil, maintaining (or even enhancing) the biodiversity of the cloud forests.

All changed with a drop in the price of coffee that made traditional practices uneconomic for all but the biggest growers. So the temptation is to replant with high-yielding varieties that need sunlight, not shade. And NAFTA will make it easier for big coffee-blenders to move in.

Faced with this prospect, some growers are moving out of coffee and into cash crop monocultures such as cane sugar. The cloud forests that once ran all along the eastern slopes of Veracruz are now

under threat. Come back in a few years, says Sergio Guevara, director of Xalapa's SEP-CONACYT Institute of Ecology, and it will all be gone.

Of course, old-fashioned coffee-growing had environmental costs; washing and processing the beans yields residue that poisons local rivers. That the polluter should pay has long been the principle of Mexico's environmental legislation, but the polluter has rarely been required to pay up. But the advent of NAFTA means enforcement, forcing the 80-member Coatepec Coffee-Processors' Association into the arms of Eugenia Olguín and her colleagues at the Institute's Environmental Biotechnology unit, which is working on effective ways to compost coffee-pulp rather than voiding it as waste.

The unit, a kind of intermediate technology outfit, looks for simple solutions to pressing but mundane problems. How, for example, to help a pig farmer, add value to the polluting by-products of his animals? Just feed the dilute slurry to a culture of cyanobacteria called *Spirulina*, which turn it into a protein source 50 times as concentrated as soya. As well as being a possible bulk animal food, *Spirulina* is full of promising biochemicals.

But Patricia Moreno, a grassland ecologist specializing in sand dunes, is more worried about the impact of tourism on the coastline. Modern Mexico has a penchant for creating huge purpose-built beach resorts such as Cancun in Quintana Roo. Where some see building sites and bathing beaches, Moreno sees a fragile strip separating the marine realm from the forest, and across which the two interact. Nobody knows what happens when the two are sundered by a solid concrete wall of waterfront property. Will each go its own way, or will both die?

Sergio Guevara himself works on that classic problem of tropical forest conservation: how to reconcile the need to maintain rain forest with ranchers' ambitions to turn it into grazing. Guevara's answer is pragmatic: find that optimal pattern of land use in which pasture can co-exist with patches, or corridors, of forest to their mutual benefit.

There is an example of such mixed land use in the botanic garden, administered by the institute as a place for public recreation and a repository for the formidable variety of local flora. Guevara is as proud of the lack of litter as the impressive collection of cycads, comprising all 45 species known from Mexico. Harrison Ford's attendants had wanted to use the garden as a suitably lush backdrop for their movie, but the request was politely deflected. □

# Getting to the bottom of top

The announcement of the probable discovery of the top quark at Fermilab in the United States is a landmark in the history of particle physics, but an expected one. It would be enlivening if the next were unexpected.

THE top quark may have been discovered at last, at Fermilab, the high-energy physics laboratory near Chicago. Last week, those attending the annual meeting of the American Physical Society at Arlington, Virginia waited in vain for a statement. But this week Fermilab announced — with proper caution — that it believes it has manufactured a handful of top quarks. The mass of the particle appears to be within 10 per cent of 174 GeV, within recently established upper and lower bounds, but higher than many had expected.

As things stand, Fermilab is the only laboratory in the world equipped to announce the discovery of the top quark; until CERN has its Large Hadron Collider running, no other place can hope to get within range.

But why all the excitement about the top quark, of whose ultimate existence nobody has much doubt? It has something to do with the principle that seeing is believing; inference, however neat the underlying theory, is always less persuasive than demonstration. But it would be also be galling for high-energy physicists to be basing calculations of the properties of particles on the assumption that the top quark exists when they had not been able to manufacture a single specimen.

Quarks are, of course, the ingredients of particles of nuclear matter such as protons and neutrons. In the standard model, they come in pairs, one pair for each of the three 'generations' that make up the family of electron-like particles, and have amusing names — 'up' and 'down', 'strange' and 'charm', 'bottom' and ... 'top', of course. Quarks cannot exist in isolation, but only in pairs (when they make mesons) or triplets (when they make nucleons).

Fermilab is wise to have been cautious. Ten years ago, the UA1 collaboration at CERN had a handful of events that seemed tantalizingly as if caused by the production and decay of top quarks. A lengthy *Physics Letter* (147B, 493; 1984) assessed the pros and cons and concluded that, if the events were due to the decay of top quarks, their mass must be between 30 and 50 GeV. But as more data accumulated, the background noise turned out to be larger than originally measured and

the handful of particles ceased to be significant.

Since then, experiments at CERN and at Fermilab have gradually pushed up the lower bound on the top quark's mass to a value of  $\geq 131$  GeV, (S. Abachi *et al.* *Phys. Rev. Lett.* 72, 2138; 1994). That lower limit is surprisingly high, some 50 per cent more than the mass of the intermediate vector boson called the  $Z^0$  and even greater than the mass of the atomic nucleus of a silver atom.

For the time being, Fermilab is the only accelerator that could directly produce top quarks with such great masses. There are two experimental teams called CDF (for Collider Detector at Fermilab) and prosaically D0, each with hundreds of members. Reaching an agreed position on the interpretation of data, and maintaining prudent or seemingly silence in the process, is fraught with problems, especially when collaborators are analysing data at institutions on three continents. And everybody is conscious of the dangers of premature claims that turn out to be false, of which there has already been a sprinkling.

Since mid-March, rumour has nevertheless been rife that Fermilab was on to something. If nothing else, graduate students have been turning in theses with samples of the data gathered at Chicago. But the barely suppressed excitement of the people there has also been significant.

So it is worth remembering that there is already good evidence for the existence of the top quark. If it is accepted (and there is no choice) that quantum field theory applies to quarks as it does to electrons, then the top quark has already manifested itself in its perturbation of precisely observable quantities at CERN's LEP, much as fluctuations of the quantum electromagnetic field determine the Lamb shift in the hyperfine spectra of atoms.

For what it is worth, the bosons  $Z^0$  and  $W^\pm$  were similarly inferred from precision data before their production at CERN. But the data so far collected at LEP with a bearing on the top quark provide only an upper limit of 200 GeV for its mass.

It is natural that particle physicists seek direct materialization of top quarks or their composites with other quarks, for the signal of top decay is expected not to be simply diagnostic. Among other pathways, for example, the decay of top is expected to yield a  $W$  and a bottom quark, each of

which will then further decay into final states, electrons and muons for example. But there are other processes, unrelated to top quarks, that can yield the same signals. Isolating the signal from the noise is the essence of the hunt for the top quark. And the more massive it is, the more difficult it must be to separate the signal from the background.

The CDF collaboration's draft paper has the positive title "Evidence for top quark production in  $p\bar{p}$  collisions at 1.8 TeV" and runs to nearly 200 pages. The evidence consists of a handful of possible 'signal' events with the characteristics of the top quark, but there are other features that do not fit exactly into the expectations, and which the paper recognizes and debates. The events, assumed real, correspond to

$$m_t = 174 \pm 10 \pm {}^{13}_{12} \text{ GeV}.$$

The statistical significance is low enough that one must allow for the possibility that subsequent data may home in on a different mass and that these events will be assigned to background or to some other phenomenon. It is a powerful measure of the caution with which the result is put forward that those concerned have written 200 pages of a full paper before boiling this down to what is expected also to be a Letter (in *Physical Review Letters*). The data are what the title says: evidence (but not proof) that the top quark exists.

How, if at all, will this development change high-energy physics? When the top is eventually agreed upon, it will indeed provide the final ingredient of the third generation of matter. High-energy physics is also awaiting the Higgs boson, and there are questions such as that of 'supersymmetry' still to be decided. They may be tasks for the Large Hadron Collider, if and when that is built at CERN.

Meanwhile, we should not overlook the possibility of being surprised. When 'strange' matter was first found in 1947, the concept of a quark had not been formulated. And the signal that led to the recognition of the tau lepton (heavy cousin of the electron and the muon) was extracted from noisy data only with dedication comparable with that which has probably now given us the top quark. "Who ordered that?", people were asking nearly twenty years ago. The same cry may yet be heard at the accelerator laboratories in the years ahead.

Frank Close & John Maddox



# A sudden squall in the cosmos

August E. Evrard and Nick Kaiser

ANCIENT Chinese astronomers would have been pleased to hear of the latest results on large-scale motions in the Universe, reported by Lauer and Postman in last week's *Astrophysical Journal*<sup>1</sup>. The Chinese believed that a "hard wind" blowing throughout the heavens kept the stars and planets suspended in their motion about the Earth. Lauer and Postman now have data that indicate the existence of a very big wind indeed. Their study suggests motions on a scale roughly one-tenth the size of the observable Universe.

The finding goes against the current orthodoxy of popular cosmological models as well as other empirical evidence, all of which points towards the Universe being a quieter and gentler place on its largest scales. So how have they arrived at this conclusion?

Their measurement follows a venerable practice in observational cosmology, the use of so-called 'standard candles'. In this case, the candles are a set of 119 brightest cluster galaxies extracted from the famous catalogue of Abell *et al.* These galaxies define a 'Hubble diagram' (apparent magnitude against redshift) with very small scatter, indicating they have very similar intrinsic luminosities. Relative motion between the observer and the Abell cluster inertial frame (or ACIF) would generate a small spatial asymmetry in this relation. It is this asymmetry that Lauer and Postman set out to find.

Imagine wallpapering the inside of a very large sphere with many identical light sources. Observing the distribution while sitting at rest at the centre of the sphere, one would, not surprisingly, see a uniform angular distribution of light. If, instead, one views the sphere from the centre while riding a rocket (or, in our case, planet) travelling with some velocity  $v$  with respect to the surrounding sphere, then a Lorentz transformation into the frame of the rocket yields the result that the sphere will appear brighter in the forward direction (parallel to  $v$ ) and dimmer in the opposite direction<sup>2</sup>. This expected pattern is evident in the cosmic background radiation<sup>3</sup>, and the natural interpretation is that the Local Group of galaxies is moving, with respect to the inertial frame defined by an isotropic microwave background, at a velocity of about 600 km s<sup>-1</sup> in a direction pointing just south of the constellation Leo.

Our motion with respect to the micro-

wave background frame (termed our 'peculiar velocity') is believed to arise from the cumulative gravitational tug of surrounding mass concentrations. Because the amplitude of clustering in the Universe decreases with increasing scale, one expects the influence of nearby, smaller mass concentrations to be more important than that of very distant, larger-scale irregularities in determining the peculiar

of isotropy of the Universe on its largest scales. The other interpretation, espoused by Lauer and Postman, is that the entire ACIF is moving with respect to the inertial frame of the microwave background. The implied velocity is  $689 \pm 178$  km s<sup>-1</sup> in a direction nearly 60° off the microwave background hotspot.

What is troubling about this interpretation is the length scale of the implied motion. The sample of galaxies covers both hemispheres away from the galactic plane to a depth of nearly 15,000 km s<sup>-1</sup> (to obtain distances in units of length, such as megaparsecs, divide by the Hubble constant,  $H_0 = 100h$  km s<sup>-1</sup> Mpc<sup>-1</sup> with  $h$  between 0.5 and 1.0). This is more than twice as deep as any previous samples used to measure bulk motions, including those that led to the proposed 'Great Attractor' a few years ago (see ref. 4 for a recent discussion). If this motion is due to the gravitational influence of a massive perturber, then the scale of the perturber must be enormous (the term 'Monster Attractor' springs to mind), equivalent to several hundred rich clusters of galaxies arranged in such a manner as to produce a fractional density enhancement of a few tens of per cent. Such a structure is unprecedented in even the largest galaxy catalogues currently available.

News of Lauer and Postman's result first surfaced over a year ago, and a meeting in Paris last July specifically devoted to cosmic velocity fields provided an initial opportunity for scrutiny by experts. Although many harboured a hope that the result was due to peculiarities in the data set, no obvious flaw in the observations was found. In their current article, Lauer and Postman go to great lengths to demonstrate the robustness of the result. Their defensiveness is not surprising: at the Paris meeting, two groups<sup>5</sup> had presented analyses indicating inconsistency between the inferred ACIF velocity and both theoretical and empirical measures of large-scale structure at greater than 95 per cent significance. The amplitude of ACIF streaming is squeezed from larger scales by measurements of the microwave background anisotropy and from smaller scales by other measures of local galaxy clustering and streaming

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

The Coma cluster of galaxies, one of the 119 'standard candles' used by Lauer and Postman to measure the motion of the Milky Way with respect to the rest of the Universe.

velocity. If the prejudice — that the Local Group's peculiar velocity is driven by irregularities well within the outer boundary of this sample — were true, then the measured velocity should be very close to that determined from the cosmic microwave background. In other words, the ACIF should be nearly 'at rest' with respect to the microwave background frame.

Lauer and Postman's precise photometry does display the expected dipole signal, but the derived velocity vector points in the wrong direction, towards the constellation Lepus which is 75° away from the microwave background hotspot. To your average cosmologist, this result was about as expected as waking up to find you've grown webbed feet and a large yellow bill where your mouth once was.

Besides sheer disbelief, there are two ways to interpret this result — one ugly and the other alarming. The ugly (though admittedly more dramatic) solution is to assume that the microwave background radiation possesses an intrinsic dipole anisotropy, which invalidates the interpretation of Local Group motion as its sole cause. Such an assumption makes a mockery of one of the fundamental principles underlying modern cosmology: that

1. Lauer, T. R. & Postman, M. *Astrophys. J.* **425**, 418–438 (1994).
2. Peebles, P. J. E. & Wilkinson, D. T. *Phys. Rev.* **174**, 2168 (1968).
3. Smoot, G. F. *et al.* *Astrophys. J.* **371**, L1–L4 (1991).
4. Courteau, S., Faber, S. M., Dressler, A. & Willick, J. A. *Astrophys. J.* **412**, L51–L54 (1992).
5. Feldman, H. & Watkins, R.; Strauss, M. *et al.* in *Cosmic Velocity Fields* (ed. Bouchet, F.) (Cambridge Univ. Press, in the press).

motions. Nonetheless, there remains a small possibility that the result is simply a statistical fluke.

In situations like this, there is no substitute for more data. And more data will certainly come, although they will take several years to gather. In the meantime, cosmologists will scramble to find ways to accommodate this new result, like picnickers sent scurrying by a sudden squall. Most would hope that, as in a thunder-

storm, the "hard wind" will eventually die away, and the Universe on large scales will be restored to the tranquil state it seemed to possess just a few short months ago. □

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## PALAEONTOLOGY

# Whales leave the beach

Michael J. Novacek

THE mammalian clade, to which we belong, primarily represents the baroque extravagance of terrestrial adaptations — burrowing, galloping, hopping, bipedal walking and tree climbing, not to mention gliding and powered flight, the probable extensions of a lifestyle in the trees. But a few mammals returned to the aquatic milieu of their vertebrate ancestors. In the case of the Order Cetacea, the whales and dolphins, this return to the sea was so decisive that adaptations to swimming, diving and feeding match or surpass those in fishes and sharks. It was a big evolutionary plunge, one that has eluded documentation for many decades.

Things, though, are changing. New discoveries of early fossil whales, such as those of Thewissen *et al.* (published in *Science*<sup>1</sup>) and Gingerich *et al.* (page 844 of this issue<sup>2</sup>), provide striking evidence for both the phylogenetic connections of cetaceans as well as the fascinating mosaic of early adaptive experiments in the transition from land to water. This expanding fossil casebook on the origins of whales is one of the triumphs of modern vertebrate palaeontology.

Intuition may weigh against the notion that something so 'fishy' as a whale may have closest kinship with land mammals. Aristotle, however, was not fooled by intuition; he pointed out that whales and dolphins, unlike bony fish, are live-bearing (viviparous). John Ray, in his classic treatise<sup>3</sup> of 1693, was more emphatic: "For as except as to the place in which they live, the external form of the body, the hairless skin [*sic*], and the progressive or swimming motion, they [cetaceans] have almost nothing in common with fishes, but in remaining [characters] agree with viviparous quadrupeds".

Modern systematists have gone some way in refining this concept. The Order Cetacea seems to be related to the great radiation of herbivorous ungulate mammals, and specifically the cloven-hoofed (even-toed) artiodactyls, an order whose modern members include deer, antelope, camels, pigs, giraffes and hippos. The

whale-artiodactyl link is supported by anatomical and molecular evidence<sup>4-6</sup>. On the morphological side, further refinement comes from the study of a group of lumbering four-legged mammals, mesonychids, that thrived in the Early Cenozoic and were especially diverse in central Asia<sup>4,7</sup>. There is a general view that mesonychids represent the intermediate lineage between whales and other ungulates<sup>7</sup> (although this allocation could stand some re-examination). At any rate, mesonychids have served as a *bauplan* for the evolutionary modifications leading to cetaceans.

Until recent years, the nature of this transition was a matter of inference based on comparison of whales with their putative terrestrial relatives. The gap was filled by a series of discoveries of fossil archaeocete whales<sup>8</sup>. These forms show small pelvic girdles and hindlimbs, whereas modern adult whales retain only vestiges of pelvic and hindlimb elements embedded in the flank musculature. Unfortunately, the adaptive role of these archaeocete hind appendages was uncertain. They seemed of doubtful use in locomotion, but might have functioned as copulatory guides<sup>8</sup>.

A leap in insight comes, however, with Thewissen and colleagues' discovery<sup>1</sup> of *Ambulocetus*, a truly remarkable fossil from 52-million-year-old sequences in Pakistan. *Ambulocetus* can be clearly allied with archaeocetes and other cetaceans, based on features of the middle ear, muzzle, skull roof and teeth<sup>1,4</sup>. Behind the skull, however, the specimen shows an extraordinary combination of features that anticipate, but do not fully embody, the aquatic adaptations of cetaceans. Both front and hindlimbs are well developed, with flexible elbows, wrists, knee joints and digits. The hand is large and elongate and the hind foot is huge. Toes end in hooves as in mesonychids and other ungulates, and the tail is notably long.

Thewissen *et al.* propose that *Ambulocetus* was amphibious; that it was possibly awkward but mobile on land, and

adept at swimming. The long tail suggests that this creature lacked a fluke, and from the shape of the lumbar vertebrae it seems that the creature swam by undulations of its vertebral column. The large paddle-like hind feet were thrust through the water when the back was flexed and extended. Unlike seals, however, the plane of undulation was not mediolateral but dorsoventral, as in living cetaceans. The forelimbs were probably involved in steering but not propulsion. It seems logical that whales may have gone through a seal-like amphibious stage early in their evolution, but the direct evidence for such a phase, as represented by *Ambulocetus*, is extraordinary.

A seaward shift beyond *Ambulocetus* is dramatically exemplified by the Eocene whale, *Rodhocetus*, now described by Gingerich *et al.*<sup>2</sup>. *Rodhocetus* is geologically younger than *Ambulocetus* and also shows a more specialized apparatus for swimming. Its short cervical vertebrae, large unfused sacral vertebrae and reduced femur are derived features associated with adeptly swimming archaeocetes and modern whales. At the same time, *Rodhocetus* retains features of the vertebral column and pelvic girdle that hark back to terrestrial relatives<sup>2</sup>. So it was not amphibious like *Ambulocetus*, but did show a mosaic of aquatic and terrestrial traits. The new specimen also casts light on early whale habitats, in that the animal was found in rocks that indicate deep-water (neritic) environments. To date, early archaeocetes have been limited to near-shore deposits, and so *Rodhocetus* demonstrates early variations in both locomotion and distribution. Indeed, the diversified lifestyles revealed by these early fossils suggest that whale evolution got off to a very quick start.

*Ambulocetus*, *Rodhocetus* and other more aquatically specialized archaeocetes cannot be strung in procession from ancestor to descendant in a *scala naturae*. Nonetheless, these fossils are real data on the early evolutionary experiments of whales. They powerfully demonstrate transitions beyond the reach of data, whether molecular or morphological, derived from living organisms alone.

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# Cajal's rational psychology

Roger A. Nicoll

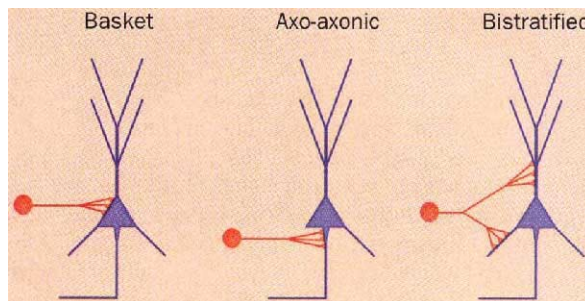
DURING the last century, investigators began focusing their light microscopes on the brain. They were driven as much by the extraordinary beauty of what they saw as by the insight they gained. The underlying premise behind this intense scrutiny of the brain's intricate cellular architecture is best summarized by the most famous of neuroanatomists, Ramón y Cajal: "The inscrutable arcanum of the organization of the brain... attracted us irresistibly. We saw that an exact knowledge of the structure of the brain was of supreme interest for the building up of a rational psychology. To know the brain, we said, is equivalent to ascertaining the material course of thought and will"<sup>1</sup>.

We may still be a long way from a "rational psychology", but the paper by Buhl *et al.* on page 823 of this issue<sup>2</sup> makes a substantial step towards providing an "exact knowledge" of the cellular building blocks of the hippocampus, a structure that is central to certain forms of learning and memory. As with nineteenth-century neuroanatomy, these results provide both beauty and insight. Using paired intracellular recordings, the authors have characterized the detailed anatomy and physiology of three distinct classes of inhibitory interneurons. As each class of interneuron has a unique role in controlling the excitability of the principal cells of the hippocampus, which in turn transmit signals to the next integrative station, the findings indicate a remarkable division of labour.

The grey matter of the central nervous system, best typified by the cerebral and hippocampal cortices, is composed broadly of two types of neurons — interneurons (local circuit neurons) and principal (relay) neurons. The interneurons, which are predominantly inhibitory, contact neurons within a local region of the cortex, whereas the principal neurons, which are predominantly excitatory, send their axons to distant regions of the brain. Untangling this cortical bramble thicket has been an exceedingly difficult task, one in which progress has been paced by technological breakthroughs. The first was the Golgi stain, which, for reasons that are still unknown, stains only a few neurons in a tissue section, but stains them in their entirety. The Golgi stain was the tool that Cajal exploited to make his vast contributions. A limitation of this method, however, is that one can only infer function; so although Cajal's record in correctly describing neuronal circuits is legendary, he had no way of knowing

whether synaptic contacts were excitatory or inhibitory.

Microelectrodes, which permit study of individual neurons in the central nervous system<sup>3</sup>, provided the tool to answer this question. Unfortunately single-cell analysis, in the conventional mode, also gives incomplete information. To get all of the necessary information one must record simultaneously from two functionally con-



The three classes of hippocampal inhibitory interneurons. The basket cell primarily contacts the soma of principal cells. The axo-axonic cell primarily contacts the axon initial segment of the principal cell. The bistratified cell primarily contacts the apical and basal dendrites of the principal cell.

nected neurons and fill each of the cells with a stain. Ideally the stain should be electron dense so that one can define the synaptic contact at the level of the electron microscope. This requires extraordinary patience, as well as determination to rival that of Cajal himself. Nonetheless Buhl *et al.*, and Gulyás *et al.* in a paper<sup>4</sup> published in December last year, have succeeded in providing just such an analysis.

In the latest work<sup>2</sup> the authors studied the functional contacts made by three classes of interneurons whose cell bodies are intermingled with those of the principal cells (see figure). The three types of interneurons have several features in common. They all release the inhibitory transmitter GABA onto GABA<sub>A</sub> receptors, and they all form up to 12 synapses with the principal cell (which ensures a high safety factor of transmission). But they each target different domains of the principal cell and exert distinct effects on neuronal excitability.

The basket cell forms synapses on the soma of the principal cell. Discharge of the basket cell causes a rapid hyperpolarization which, in some cases, is followed by a rebound discharge. Because a single basket cell contacts hundreds of principal cells, this rebound discharge provides an ideal way of synchronizing large populations of cells, in addition to the direct and powerful inhibition that it exerts.

The axo-axonic cell forms synapses only on the axon initial segment of the principal cell. The initial segment has the lowest

threshold for generation of action potentials in the cell, and therefore the axo-axonic cell is ideally suited to control the discharge of the principal cell. There is a convergence of about six axo-axonic cells; however, because of the small diameter of the initial segment it is likely that the input of just a single axo-axonic cell would have a powerful influence on the excitability of the principal cell.

The bistratified cell forms synapses on the basal and apical dendrites of principal cells, which is where excitatory synapses of Schaffer collaterals are made. This convergence suggests that the bistratified cell controls local excitatory inputs to the cell. More specifically, the slow time-course of the inhibition evoked by this class of interneuron is remarkably similar to that of the NMDA receptor-mediated synaptic response. The NMDA receptor, which can function only when the cell is depolarized, is essential in initiating long-term changes in synaptic strength. Thus both the spatial and temporal properties of the bistratified cell inhibitory input indicate that this interneuron is crucial in determining which excitatory synapses will undergo changes in synaptic strength.

The new results<sup>2</sup> indicate an extraordinary division of labour among the different classes of inhibitory interneurons, which by contacting different domains of the principal cell have distinct roles in sculpting its activity. This clear separation of function suggests that each type of cell might be controlled independently. Such control seems to be achieved in two ways.

First, the dendritic tree of the different types of interneuron samples different regions and therefore different subsets of excitatory afferents (Schaffer collaterals and so on). Second, there is evidence that different classes of interneurons can be distinguished by the types of neurotransmitter receptors that they possess. For instance, a few interneurons receive a dense plexus of serotonin-containing fibres<sup>5</sup>; and a subset of interneurons,

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perhaps the same ones, express the 5-HT<sub>3</sub> receptor<sup>6</sup>, which, when activated, strongly excites the interneuron<sup>7</sup>. It also seems that different interneurons can have different mixes of both ionotropic<sup>8</sup> and metabotropic<sup>9,10</sup> glutamate receptors. Given the rich variety of neurotransmitter fibre systems (noradrenergic, cholinergic, opiodergic, to name just a few)<sup>11</sup> that converge on the hippocampus, it is easy to imagine how different sets of interneurons could be selectively engaged in different behavioural states. In this context it will be of interest to define the pharmacological fingerprint of the three classes of interneurons described by Buhl *et al.*

Finally, a recent report shows that one can record in the behaving animal from large populations of hippocampal neurons, including interneurons, for prolonged periods<sup>12</sup>. With refinements in this technique, it may be possible to correlate the firing of each class of interneuron with a specific aspect of the animal's behaviour. Perhaps we are not, after all, that far away from Cajal's rational psychology. □

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## CRETACEOUS/TERTIARY BOUNDARY

# Blind tests and muddy waters

Jan Smit

THE idea that the Earth suffered an impact from a large extraterrestrial body at the Cretaceous/Tertiary (K/T) boundary 65 million years ago is now almost unanimously accepted, to judge from a recent conference in Houston\*. Less consensus exists about the damage that might have been inflicted on the biosphere. Some researchers contend that the impact did occur, but had little, if any, influence on the biosphere. At the opposite extreme, others maintain that all extinctions at the K/T boundary were caused by the impact effects, taking place within a few years.

Neither is true. Inoceramid and reef-forming rudist clams disappeared some millions of years before the K/T boundary. But microorganisms such as planktonic foraminifers and coccoliths show a worldwide crash in abundance exactly at the impact layer, at both low and high latitudes, although the final extinctions may have lingered on for a while.

At the conference, the undoubted highlights were the extensive data from the Chicxulub crater thought to be the site of the impact, and from the ejecta and coarse deposits (generated, it is thought, by a tsunami or giant 'tidal wave' created by the bolide's offshore impact) now found at the K/T boundary all around the Gulf of Mexico from Alabama to Guatemala.

Two debates punctuated the meeting. The first was the question of gradual versus sudden species extinctions at the boundary, and was addressed by 'blind tests'. Under the supervision of R. N. Ginsburg (Univ. of Miami), researchers had been given coded samples of crucial K/T sections from Tunisia and Italy; at the meeting, A. G. Fisher (on behalf of Ginsburg) reported what they had found. The second concerned the interpretation

of sandstone beds at the K/T boundary.

The first of the blind tests was, among other things, aimed at testing whether iridium anomalies were present in clay layers near Gubbio, Italy, other than in the K/T boundary clay. The controversy now seems to have been settled: no iridium was found in these layers (F. Asaro, Lawrence Berkeley Laboratory; J. H. Crockett, McMaster Univ.; U. Krähenbühl, Univ. of Bern; H. T. Millard, US Geological Survey, Denver; C. J. Orth, Los Alamos National Laboratory; R. Rocchia, CNRS, Gif-sur-Yvette).

The second was designed to test extinction patterns across the K/T boundary. This test was carried out in the type locality of the boundary, the El Kef section in Tunisia.

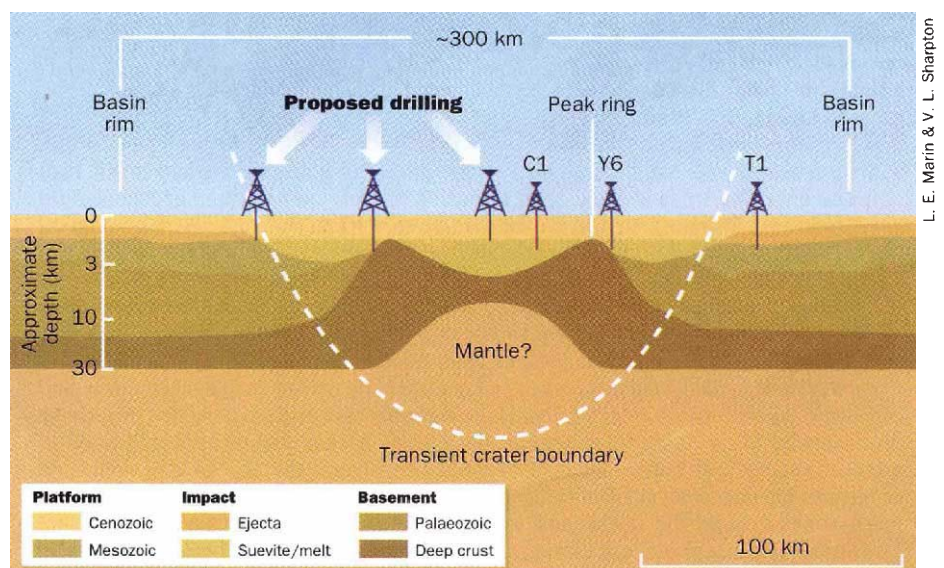
When I looked at this question in 1982, I concluded<sup>1</sup> that there were no significant plankton extinctions before the K/T

boundary, a major mass-extinction event at the boundary, and some, unimportant, survivorship above it. In contrast, Keller<sup>2</sup> reported a stepwise extinction record beginning well before and continuing across the K/T boundary. The new test therefore concentrated on extinctions before the boundary, because substantial extinctions just before the event might cast doubt on the linking of a single impact to the extinctions, or suggest that Chicxulub was part of a larger impact shower.

Four micropalaeontologists were given equal splits of six (coded) samples, three from 75 cm to 6 cm below and three from 7 cm to 85 cm above the K/T boundary. They returned a range chart of all species encountered, and an estimate of their abundance (X. Orue-etxebarria, Univ. Pais Vasco; R. K. Olsson, Rutgers Univ.; B. Masters, Sapolpa, Oklahoma; J. I. Canudo, Univ. Zaragoza).

And the results? Each individual analysis taken on its own shows species disappearances before, at and after the K/T boundary. But when the charts from the four participants were lumped together, they did not demonstrate any disappearances before the K/T boundary. Presumably the Signor-Lipps effect is to blame here: it is unlikely that rare species will be found in all samples. Keller's original analysis<sup>2</sup> probably suffered from the same effect: it is hard to find rare species, and the dissolution of planktonic foraminifers in the topmost Cretaceous sample makes the task still harder.

The second debate revolved around the interpretation of sandstone beds at the K/T boundary in outcrops around the Gulf of Mexico from Alabama to Chiapas. Are these made up of ejecta and local material stirred up and rapidly redeposited by impact-induced tsunamis<sup>3-5</sup>? Was the material instead washed from land by tsunamis beating on the shore, and de-



Schematic cross-section through the Chicxulub structure, showing the positions of the old cores C1, Y6 and T1, and the proposed new drilling sites.

\*New Developments Regarding the K/T Event and Other Catastrophes in Earth History, Houston, Texas, 9-12 February 1994.



posited as a single turbidite layer (B. F. Bohor, US Geological Survey)? Or are the beds a sequence of turbidites caused by storm waves at a sea-level lowstand, and unrelated to the impact<sup>6</sup>? Tsunami deposition of the entire complex would take only a few days, the alternative mechanism tens of thousands of years.

A pre-meeting field trip offered the chance to settle the issue. There, several sedimentologists assessed the K/T sandstone deposits as highly unusual, something they had never seen before (R. H. Dott, Univ. of Wisconsin; E. Clifton, Conoco, Texas; D. Lowe, Stanford Univ.;

A. Ekdale, Univ. of Utah). The sands themselves had been transported by powerful currents which ran in several directions, and had been deposited rapidly.

If a significant amount of time had passed between deposition of individual sand layers, burrowing organisms should have colonized the surfaces, and there would have been traces of burrows. None are present with the exception of the topmost surface of the sandstone layers, demonstrably colonized after its deposition. The field-trip participants concluded that, although they could not definitely

exclude deposition in thousands of years, nothing indicated that these sandstones had been laid down over a prolonged period of time.

Some reports stressed that in the Gulf coast area, the iridium maximum taken to mark the K/T boundary is in a mudstone between 5 (el Mulato) and 30 cm (Brazos River), well above the sandstone layer (D. Beeson, Chevron, New Orleans). This would imply that the sandstones date from before the impact. But this mudstone, which tops the sandstone and contains several iridium peaks, gradually decreases in grain size upwards (Smit). Most probably the mudstone represents the slower settling of the muds and silt stirred up by the tsunami waves. The fine, cosmic, iridium-rich dust settled together with these muds.

The widely accepted story that the missing cores drilled from the Chicxulub crater in the 1960s were lost in a warehouse fire has now been unveiled as a myth (V. L. Sharpton, Lunar and Planetary Institute, Texas). Most are still available in Mexico. The Ticul-1 well cores from just outside the crater rings (see figure on previous page) contain a polymict breccia more than 1 km thick. This is a suevite breccia with altered meltclasts, similar to the breccias drilled within the crater (Yuc-6). Other cores outside the crater contain the same suevite breccia. The breccia components seem compatible with the Yucatan basement: they include gneisses, schists, granite and red sandstone and shale fragments. Many of these fragments contain quartz and feldspar crystals with uncontested shock deformation lamellae (Sharpton; A. R. Hildebrand, Geological Survey of Canada).

Overall, evidence from these cores supports the impact theory rather than, say, a volcanic origin for the Chicxulub structure. But as the cores are not continuous, important information about basement, melt sheet and the first sediment infilling of the crater is still lacking.

A Mexican consortium started drilling in the Chicxulub structure in February (L. E. Marín, personal communication). Their goal is to drill one deep hole just outside the central ring structure, penetrating the melt sheet to reach fractured, uplifted basement, and to drill several shallow holes in the outer rings. It is expected that cores will shortly become available to the scientific community. □

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## SOIL SCIENCE

### Californian acid rock

CALIFORNIA abounds in thick forests and barren deserts, but they are not usually found side-by-side as in this photograph from the northern Californian Klamath mountains. The fact that swathes of healthy coniferous forest in this region are interrupted by bare patches of ground with unusually acidic soil has caused no little perplexity amongst soil scientists and ecologists. Now it seems that the rocks themselves may be the culprits.

According to Randy Dahlgren (page 838 of this issue) the bedrock in these mountains contains large amounts of nitrogen, bound up as ammonium cations. In the forested areas, weathering has gradually removed much of this ammonium from the upper soil layers. But Dahlgren's theory is that for some parts of the forest, a contained disturbance (for example a small forest fire) stripped away the trees and exposed the soil to the elements. Rapid erosion would have followed, bringing to the surface relatively fresh, nitrogen-rich geological material. This would then have been oxidized, giving large amounts of nitric acid. The soil acidity would have caused intense leaching of nutrient cations needed for soil fertility. It would also have mobilized potentially toxic levels of aluminium. All in all, the environment would become rather unfriendly to aspiring saplings, and this, says Dahlgren, can explain the lack of vegetative regrowth.

By contrast, in the healthy forest organic acids bind up any rogue aluminium, and the litter layer replenishes any nutrient cations lost to the soil. But for these mechanisms to come to the rescue of the barren areas, new trees would need to grow; it seems that the initial loss of vegetation traps the soil in a vicious and unrelentingly barren cycle. Dahlgren points out that geological nitrogen is found in many rocks around the world, and wonders if it could be having a similar effect elsewhere. He also points out that regions such as this one could prove to be useful analogues for the damage that can be done to ecosystems from anthropogenic nitrogen, deposited not from below, but from above.

Gabrielle Walker



# Shellfish genes kept in line

Laurence D. Hurst and Rolf F. Hoekstra

THE received orthodoxy<sup>1</sup> that animal mitochondrial DNA (mtDNA) is maternally transmitted was given a severe blow a few years ago with the demonstration that in mussels (*Mytilus*) it apparently comes from both parents<sup>2-4</sup>. Two reports, by Skibinski *et al.*<sup>5</sup> and Zouros *et al.*<sup>6</sup> on page 817 of this issue, now reveal that although the orthodoxy must indeed be re-evaluated, one set of theories attempting to account for maternal transmission may yet remain intact.

Paternal transmission of animal mtDNA has been described in both mouse and *Drosophila* hybrids (see ref. 2 for references). These instances, however, are not greatly troubling as the leakage is limited and individuals are not adapted to the hybrid condition. Paternal leakage has also been reported in drosophilid intra-specific crosses<sup>7</sup>, but here again it is very limited.

Mussels, though, are different, as the level of paternal transmission is high even in intra-specific crosses. Two different mitochondrial genomes, *F* and *M*, exhibiting about 10–20 per cent sequence divergence, can be detected<sup>3,4</sup>. Curiously, a large sex difference in the probability that an individual may have copies of both *F* and *M* is reported<sup>4,6</sup>. Whereas females usually (if not always) have only the *F* genome (hence the name), most males harbour both *M* and *F* genomes<sup>4</sup>. How could this be so? If the genomes are biparentally transmitted, there should be no difference between the sexes.

Skibinski *et al.* and Zouros *et al.* now provide evidence for a solution to this paradox. Both teams report that the transmission of mitochondrial types is dependent upon the sex of the offspring (see figure). Sons receive *M*-type mitochondria from fathers and *F* type from their mothers. Daughters in contrast usually receive only the *F* type. Because there is only a very low level of *F* mitochondria in sperm it is assumed that all, or nearly all, of a daughter's mitochondria must be maternally derived. Neither group has found direct evidence of paternal transmission of *F*.

How does this finding support a theory that predicts the evolution of maternal inheritance? One advantage of maternal inheritance is that it may prevent the spread of deleterious cytoplasmic genomes<sup>8-14</sup>. Consider a mutant mito-

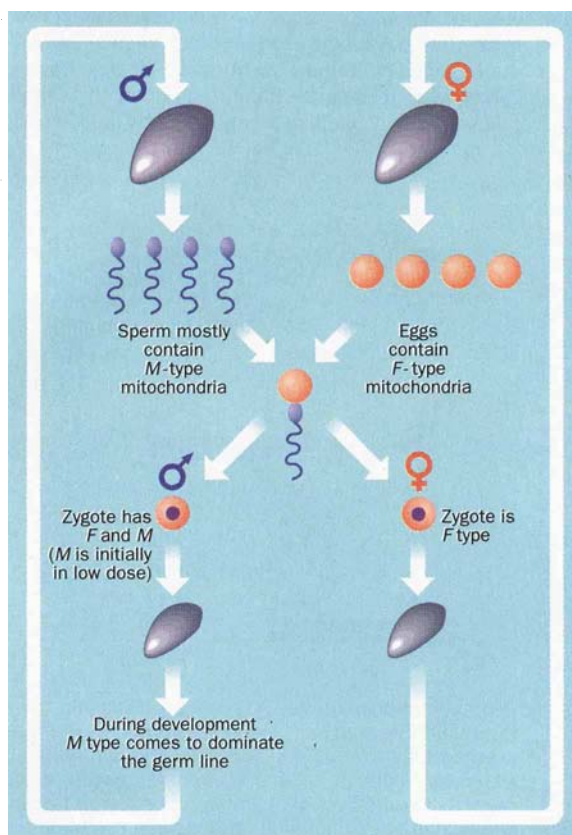
chondrial genome that, for example, could replicate faster than competitor genomes; it might do this because it has a deletion and hence may easily be del-

long as the deletion is not too deleterious). Imagine, for instance, the fast replicator starting out in one individual. As it can replicate rapidly it may become over-represented in the germ cells and hence in the progeny as well. All of these progeny in turn will transmit it at a high rate and so on. The spread of the deleterious genome due to its over-representation qualifies the fast replicator to be termed a 'selfish gene' or 'selfish genetic element'.

If, however, transmission is maternal, then although selection within an individual may favour the fast replicator, it will not result in its spread through the population. The genome can only ever be found in the maternal line in which the mutant first originated. Other maternal lineages will have fitter mitochondria and so selection at the level of the individual will favour these lines.

Central to the maternal-transmission hypothesis is the idea that a newly arising mutant should not be able to move outside one uniparental transmission lineage. Typically this means a maternal lineage, but exclusively paternal transmission is equally efficient. As both Skibinski *et al.* and Zouros *et al.* point out, the *Mytilus* data actually support the view that uniparental inheritance is a defence against selfish cytoplasmic genomes. *Mytilus* mitochondria are functionally differentiated into not one but two uniparentally transmitted lineages: *F* mitochondria are transmitted down a maternal lineage, *M* mitochondria down a paternal line. Although sons receive *F* mitochondria it seems that they are not transmitted. Our preliminary models confirm that a selfish shellfish mitochondrial genome could not spread because it would be contained within one of these two uniparental transmission lineages.

In principle, any theory for uniparental transmission is vindicated by the *Mytilus* data. But the fact that *M*-type mito-



Transmission of mitochondria in *Mytilus*. *F* mitochondria are transmitted by mothers to sons and daughters; *M* mitochondria by fathers to sons. There may also be limited transmission of *F* by fathers to daughters. Any given *F* genome has its ancestry defined through a maternal line. Tracing back the history of *M* mitochondria will reveal them to be transmitted down a paternal line. The figure depicts an inbred lineage but this is just for illustrative purposes and is not intended to represent the natural condition.

chondrial. Nevertheless, because it can replicate faster than competitors it can spread in a sexual population just so long as transmission is biparental (and so

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dria come to dominate the germ line of males, despite the low initial dose<sup>4</sup>, is consistent with the notion that the *M* type is a fast replicating genome<sup>4</sup> and hence indicative of the involvement of selfish genes. Furthermore, the alternative hypotheses for the evolution of uniparental transmission require that transmission is equivalent to inheritance. The *Mytilus* data run counter to these hypotheses. It has, for instance, been proposed that uniparental inheritance evolved to prevent recombination between mitochondrial genomes<sup>15</sup>. But surely *F* and *M* could potentially recombine as they share the male germ line. Similarly, there has been emphasis on the lack of selection against uniparental inheritance rather than factors favouring it, hence it has been argued that uniparental inheritance is often a side product of other evolutionary events<sup>16</sup>. The lengths *Mytilus* has gone to must surely be redolent of selection in favour of uniparental transmission.

It remains to be worked out in detail just how sex and mitochondrial inheritance are co-ordinated in *Mytilus*. Could *M*-type mitochondria actually determine masculinity? Cytoplasmically transmitted factors can affect sex determination in animals (see ref. 17 for review) although there is no precedent for a mitochondrial effect. Or is sex determined independently of mitochondrial type? If so, the acceptance of *M* mitochondria may be but one phenotype of males. Although the data are not yet in, the detection of males without *M* type (E. Zouros, personal communication) suggests that cytochrome cannot be the unique determiner of sex.

This example provides support for the theory linking uniparental transmission of mitochondria and selfish genetic elements, but other possible examples of biparental inheritance of mitochondria in animals loom on the horizon<sup>18</sup>; furthermore, in plants<sup>19</sup> (and other non-animal lineages) there are several exceptions to the rule of uniparental inheritance. How can these be explained and why is the inheritance of cytoplasmic genomes stricter in animals than it is in plants? One probable difference between plant and animal mitochondrial genomes is a much higher mutation rate in the latter<sup>20</sup>. Perhaps then, although the level of inbreeding may explain some variation<sup>13,19</sup>, that uniparental inheritance in animals is that bit stricter may be because the rate at which potentially selfish genomes are created is that bit higher. □

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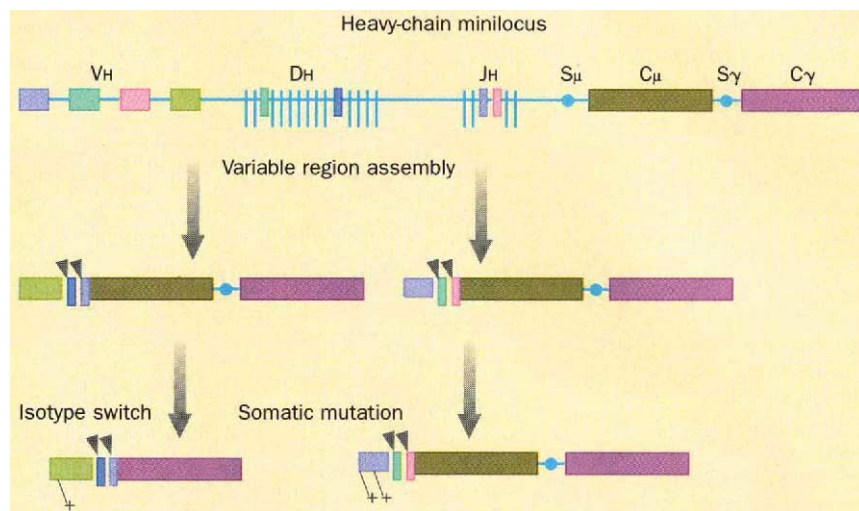
## Success in specification

Sherie L. Morrison

THE enticing goal of producing totally human antibodies with desired specificities has remained elusive. But two groups — Lonberg *et al.* writing on page 856 of this issue<sup>1</sup> and Green *et al.* in the May issue of *Nature Genetics*<sup>2</sup> — now describe mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In both cases this has been accomplished by in-

variable region is expressed joined to different heavy-chain constant regions, providing antibodies with the same specificity but different effector functions.

The mice of the two groups have been produced so that several human variable regions for both the heavy and light chain are available for expression. The multiple light-chain variable regions can be joined to different J regions, and the multiple



Representation of the heavy- and light-chain miniloci in the mice produced by Lonberg *et al.*<sup>1</sup>. At the human heavy-chain locus in the mice there are four *VH* regions, 15 *DH* elements, 6 *JH* segments as well as the genes for the  $\mu$  (*Cμ*) and  $\gamma 1$  (*Cγ*) constant regions. Combinatorial assembly of the *VH*, *DH* and *JH* elements generates considerable antibody diversity. Additional diversity results from N-region addition (arrow heads), and somatic mutation (+). These mice can also express the same variable region with differing constant regions by isotype switching using the  $\mu$  and  $\gamma$  switch regions (*Sμ* and *Sγ*). Similarly, diversity is generated at the light-chain minilocus by the combinatorial assembly of the four *VL* with the five *JL*; the light-chain locus has only the  $\kappa$  constant region gene. Random expression of different light chains with different heavy chains provides a further source of diversity.

serting elements of the human heavy- and light-chain locus into mice in which production of endogenous murine heavy and light chain was disrupted. The resulting mice can synthesize human antibodies specific for human antigens, and can be employed to produce hybridomas making human antibodies. Use of mice in which the heavy chain and  $\kappa$  light chain have been knocked out guarantees that most of the antibodies made by the immunized mice will be human.

Active immunoglobulin genes are somatically assembled from several genetic elements. In light chains, a variable region is joined to a J region. Heavy chains are assembled from variable regions, D regions and J regions. The combinatorial variety of this process contributes to antibody diversity, as does the insertion of non-germline-encoded nucleotides (N sequences) and somatic mutation of the rearranged genes. During the immune response, the specific

heavy-chain variable regions can be joined to multiple *Ds* and *Js*, with N-region addition. Furthermore, Lonberg *et al.*<sup>1</sup> show that somatic mutation takes place in the transgenic human heavy-chain variable region. So these mice have the essential equipment for generating extensive antibody diversity, and immunized animals have been used to produce hybridomas expressing specific human antibodies of reasonable affinity.

The constant region of the antibody determines its biological properties and effects; no differences in antibody functional properties have been attributed to the light-chain constant region. In Green and colleagues' mice<sup>2</sup>, only the  $\mu$  heavy chain contributes significantly to the circulating antibody population, and these mice are unable to undergo the isotype switching characteristic of the mature antibody response. But in the mice of Lonberg *et al.*, the heavy-chain locus contains both a  $\mu$  and  $\gamma 1$  heavy chain with

switch sites, and these authors show that class switching occurs. So they have reconstituted the essential part of a human-antibody-producing response in a mouse, and the system could be used both to study control of antibody production as well as to produce specific human antibodies. It is interesting, incidentally, that the mouse can furnish any additional signals necessary for the expression of the human structural genes.

How, though, does this approach compare to other ways of producing antibodies for therapeutic use *in vivo*? Among other things, antibodies have potential use as diagnostic and therapeutic agents in malignancy, infectious disease and organ transplantation. The competing technologies include genetic engineering approaches involving chimaeric antibodies<sup>3</sup> with murine variable regions joined to human constant regions, and 'humanized' (CDR grafted) antibodies with only the complementarity regions (CDR) of the murine antibody transferred to the human backbone<sup>4</sup>, and phage display systems<sup>5,6</sup>.

Large numbers of chimaeric and humanized antibodies have now been described and a few are making their way into the clinic. In most cases the recombinant antibody retains the affinity of the murine antibody and this approach can take advantage of the large number of specific murine hybridomas that have been produced and well characterized. A potential drawback is that the resulting antibodies retain murine sequences (in the case of the chimaeras the entire murine variable region; in that of the humanized antibodies, the murine CDRs and any other residues of the murine framework necessary to maintain specificity). What remains to be seen is whether these persisting murine sequences will prevent successful application of these antibodies in therapy *in vivo*, and whether they will be significantly more immunogenic than all human antibodies — even completely human antibodies contain idiotypic and allotypic determinants that are potentially recognized as foreign.

Phage-based antibody-screening systems, especially those using surface expression of antibody-binding specificities on filamentous phage, are of proven power. One way of making specific human antibodies starts with a murine antibody of the desired specificity. One of the murine variable regions is expressed paired with a phage library of variable regions for the other chain, and a human antibody of the appropriate specificity is identified. The human variable region capable of encoding an antibody of that specificity is then paired with a second phage library and screened for binding specificity. By sequentially replacing first one variable region then the second with human sequences, a murine binding speci-

ficity can be recovered in a human antibody. Alternatively, a human variable-region phage library can be screened directly for VH and VL combinations of the right specificity. Methods are now available for producing libraries of increasing complexity, and these libraries can yield specific antibodies of high affinity. Phage libraries can be screened within a matter of days; immunizing transgenic animals to produce specific antibodies and hybridomas requires months. It remains to be shown if one system can produce unique binding specificities.

Although attention usually centres on the binding specificity of a therapeutic antibody, its constant region can also alter its effect *in vivo*. As clinical data begin to accumulate, examples have emerged in which the same binding specificity will result in a useful therapeutic outcome when joined to one isotype, but not when joined to another. So we need to know more about how the constant regions influence antibody effects *in vivo*.

There are now several ways of producing recombinant antibodies, but we have yet to find out what is the main factor limiting their successful use in therapy. If it is immunogenicity, the completely hu-

man antibodies from either the phage or mice should be an improvement. If it is specificity, there are several trade-offs and well-characterized murine antibodies may have some advantages. Increasing affinity does not necessarily correlate with increasing specificity, and high-affinity antibodies may in fact show greater cross-reactivity with nonspecific antigens. But immunogenicity and specificity may not be the limiting factors. Instead it may be the effector function and biological properties of the antibodies — if this is indeed the case, the transgenic approach may be more difficult to apply than methods based purely on genetic engineering. □

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#### ATOMIC PHYSICS

## New light on antiprotonic atoms

R. J. Hughes

It was in 1947 that Fermi and Teller predicted<sup>1</sup> the existence of electromagnetically bound systems of oppositely charged elementary particles other than the common electron–proton combination. Of the many species of exotic atom that have been produced since then, the most interesting are those whose structure can be probed by spectroscopic means. Last year saw the production at CERN of gas-phase, metastable antiprotonic helium atoms (a helium atom with an antiproton substituted for one of the electrons)<sup>2</sup>, and just a year later, laser spectroscopic measurements on the system are starting to emerge<sup>3</sup>.

High-precision two-photon spectroscopy of positronium (the electron–positron atom) and muonium (the bound state of an electron and a positive muon) has produced some of the best tests of quantum electrodynamics. One might think that exotic atoms in which an antiproton replaces an electron would be similarly appealing. Typically, however, the annihilation lifetime is only a few hundred orbital periods long (a few picoseconds), and until now this has limited spectroscopy to observations of antiprotonic X-ray emission lines. (The potential for high-precision spectroscopic measurements is one of the motivations to

produce the doubly exotic antiproton–positron bound state, known as antihydrogen<sup>4</sup>, which would annihilate less rapidly.)

But the recent experiments — first in liquid helium<sup>5</sup> at KEK, the Japanese high-energy physics laboratory, then in gaseous helium at CERN's Low Energy Antiproton Ring (LEAR)<sup>2,3</sup> — show that metastable, Rydberg states of antiprotonic helium do exist with the long lifetimes required for high-precision spectroscopy. In this 'atom', the orbital speed of the antiproton in a Rydberg state is so slow in comparison with the electron's that both the antiproton and the helium nucleus can be treated within the Born–Oppenheimer approximation — so we could regard it instead as an exotic molecule with nuclei of charges  $+2e$  and  $-e$ . (In fact, Yamazaki *et al.* dubbed it an 'atomcule'<sup>2</sup>.)

According to conventional wisdom, an antiproton with a kinetic energy of a few megaelectronvolts encountering helium at standard temperature and pressure almost never annihilates immediately, but loses energy by scattering off atomic electrons (the Bethe–Bloch process), until within a few tens of nanoseconds its kinetic energy drops below the atom's ionization potential, low enough for it to replace an atomic electron and form an exotic atom.



Then the antiproton's fate should be sealed: within a few picoseconds it will reach an *s*-state by means of rapid radiative and Auger transitions (ejection of the second electron) followed by collisional Stark mixing, from which annihilation on the atomic nucleus occurs. However, in the PS205 LEAR experiments some 3 per cent of the 5-MeV antiprotons that were directed onto pure helium targets survived for several microseconds<sup>2</sup>, far longer than this argument suggests. This anomalous lifetime, which was not seen in other noble gases, was dramatically reduced in the presence of small quantities of impurity atoms, and was 14 per cent smaller in <sup>3</sup>He than in <sup>4</sup>He, suggesting that the enhancement occurred after the antiproton was captured.

Similar anomalous lifetimes had been observed with negative pions and kaons in liquid helium bubble chambers in the 1960s, and were attributed to the formation of long-lived atomic states by Condo<sup>6</sup> and Russell<sup>7</sup>. Russell had even proposed that this phenomenon could be investigated with the intrinsically stable antiproton instead of the unstable mesons, but his suggestion was not pursued until the kaon lifetime anomaly was rediscovered in 1988.

In Condo and Russell's picture, an antiproton captured by a helium atom will preferentially occupy an orbit with the same radius as that of the 1s electron that it replaces. Its principal quantum number will therefore be  $n = (M^*/m_e)^{1/2} \approx 38$ , where  $M^*$  is the antiproton's reduced mass. Some antiprotons will be captured in high-angular-momentum Rydberg states ( $l \sim n - 1$ ), for which the radiative lifetime is a factor of  $(m_e/M^*) n^3 l^2 \approx 3 \times 10^4$  larger than typical electronic radiative lifetimes (these are about 100 ps), in agreement with the PS205 observations. Moreover, Auger transitions are strongly suppressed because the ionization energy of the electron ( $I_0 = 24.6$  eV) requires a change of the antiproton's principal quantum number (and hence also its angular momentum) by six units. Thus, the exotic atom remains electrically neutral during the radiatively dominated first phase of its de-excitation, which suppresses Stark mixing and ensures that the antiproton survives up to 30 million times longer than otherwise expected.

The intuitive picture of Condo and Russell has been developed in several calculations<sup>8-10</sup> which show that there is a spectrum of such metastable antiprotonic states in the vicinity of  $(n, l) = (38, 37)$ , together with nearby levels that have short Auger lifetimes. Morita *et al.* have now provided striking confirmation of this picture. Their latest experiment<sup>3</sup> involves laser excitation of the electric dipole transition from the metastable  $(n = 39, l = 35)$  level to the Auger-dominated  $(n = 38, l = 34)$  state. Forced annihilations are

observed when an on-resonance laser beam illuminates the helium target 1.8  $\mu$ s after entry of an antiproton. Modelling the system as a simple Bohr atom indicates that the energy of this transition is about 2 eV, and the model predictions for the wavelength of the emitted photon are close to the measured value of  $597.259 \pm 0.002$  nm.

From the observed decay time of the forced annihilations a 7-ns lifetime can be estimated for the lower level, which is again in good agreement with the calculated Auger transition rate to the  $(n = 32, l = 31)$  state of the positively charged antiprotonic helium ion. Annihilation then follows within picoseconds via Stark mixing. The decay time sets a bound on the width of the transition that is about an order of magnitude smaller than the measured value of  $\Delta\lambda/\lambda = 3 \times 10^{-5}$ , where  $\lambda$  is wavelength.

However, the models indicate that it should also be possible to drive transitions between two metastable states, such as  $(n = 39, l = 36)$  to  $(n = 38, l = 35)$ , which would therefore be several orders of magnitude sharper. Assuming that these transitions are subsequently observed, antiprotonic helium will be amenable to spectroscopy with the high precisions attained on hydrogen, positronium and muonium.

Spectroscopy of metastable antiprotonic helium will surely lead to advances in precision measurements of fundamental constants and tests of discrete symmetries, such as charge-parity-time (CPT). Perhaps a precise value of the 'antiprotonic Rydberg constant' could be determined — from this, one might extract the antiproton's mass (and charge) with a precision rivalling that of ion trap measurements. If hyperfine structure could be measured, we could deduce a new value of the antiproton's magnetic moment, which is currently only known with a precision of a few parts per thousand.

On a more prosaic level, physicists can entertain themselves with the speculation that laser-driven cycles among the metastable states might enable antiprotons to survive in proximity with matter for long periods of time. The PS205 collaboration is now working towards a systematic study of the metastable state spectrum. □

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## Quick Release

As currently practised, toilet training is entirely negative. It is concerned to stop, or at least delay, the reflex emptying of the bowel. Only a fortunate few ever learn the converse skill, that of emptying the bowel at will. The rest of us must depend on artificial aids such as purgatives, which take hours to work.

And yet, in circumstances of sudden desperate fear, human beings and many other animals can empty the bowel instantly and automatically. This reflex must have evolved to reduce our weight, making it easier to run away, and possibly to make the path slippery for a pursuer. The mechanism of such automatic reactions is usually not nervous but hormonal. Fear pumps some hormonal alarm signal into the bloodstream; all over the body, local receptors react to it and prepare for the sudden emergency. The hair stands on end, the heart races, the muscles tense and the bowels loosen.

So, says Daedalus, constipation could be cured by striking sudden terrible panic into the bowels of the patient. The same result could be obtained less drastically by injecting the hormonal alarm signal itself, if it could be isolated. A variant that acted on the bowels alone, without spreading panic throughout the body, would be even better. Daedalus is now in search of it.

DREADCO's virtual-reality experts are creating computer-simulated 'virtual predicaments' of appallingly realistic horror. Brave constipated volunteers are being subjected to these bowel-loosening ordeals, while the hormonal contents of their blood are extracted and identified. Synthetic analogues will then be tested on cultures of bowel and other tissues. From the results, a drug will be designed to bind to the bowel receptors, but not to any of the other fear receptors dotted around the body.

The final product, DREADCO's 'Quick Release', will trigger immediate defecation, but no other manifestation of fear. It will give us all a wonderful new control over our unruly bodies. Divers about to climb into deep-sea gear, pilots of small aircraft about to take off, prospective long-distance bus travellers, nervous examination candidates and visitors to towns badly supplied with public lavatories, will all welcome the ability to 'dump the tanks' while it is still feasible. A prolific source of personal embarrassment will be ended; constipation will vanish overnight; urban dog owners and nurses of bed-ridden patients will gain new control of their hapless charges. But a terrible new practical joke will be born: the secret spiking of the victim's food or drink with Quick Release.

David Jones

# Ancient neurofibromatosis

SIR — Pre-mediaeval descriptions of neurofibromatosis type 1 are unknown. Our extensive review of early artworks from many civilizations has revealed possibly the earliest known model of neurofibromatosis in the form of a statue from Hellenistic times.

Neurofibromatosis type 1 (NF1), or von Recklinghausen neurofibromatosis, is an autosomal dominant condition characterized by multiple tumours including skin neurofibromas and brain gliomas. The earliest convincing medical descriptions occurred in the eighteenth century, pre-dating von Recklinghausen's classic monograph<sup>1,2</sup>. Earlier portrayals may occur back to mediaeval times<sup>2,3</sup>.

The scarcity of pre-Renaissance models of NF1 is surprising from a clinical and genetic standpoint. The skin manifestations make NF1 one of the most externally distinctive inherited diseases, surely of great fascination to early physicians and scholars. NF1 is also one of the most common inherited diseases, with a frequency of 1 in 3,000–4,000, and affects all ethnic types. Rarity in early civilizations is unlikely for several reasons. First, the prevalence of NF1 has remained steady, with the high rate of spontaneous mutation balancing reduced biological fitness<sup>4</sup>. Second, there is no founder effect (unlike cystic fibrosis, for which the initial mutation can be traced back). Finally, there is phylogenetic evidence that the NF1 gene, and therefore the disease itself, is ancient. The NF1 gene is believed to be a tumour-suppressor gene and may be crucial in growth regulation<sup>5</sup>. It contains critical regions that are highly conserved throughout the animal kingdom and in yeasts<sup>6</sup>. We undertook a survey of antique iconographic illustrations, art recordings and ancient medical texts, and have found a statue dating from the Hellenistic era that may represent the first known medical depiction of NF1.

Our statue appears as a photograph in ref. 7 showing a healthy muscular male with multiple skin nodules distributed over the entire visible torso and limbs. The nodules are smooth and sessile, not pedunculated. The limited differential diagnosis includes multiple angioliomas, familial epidermal cysts or lipomas; metastatic carcinoma, skin malignancy, leprosy, syphilis and onchocerciasis. The first three conditions are extremely rare — although lipomas are common, multiple forms as prolific as seen on the statue are exceptional. This person does not appear

to be wasted from malignant disease. Furthermore, the skin lesions neither have the correct appearance for leprosy or syphilis nor typical distribution for onchocerciasis (mainly over bony prominences). Both on appearance and likelihood of disease occurrence, the model is most representative of NF1.

The statue as it appears is labelled as a votive offering<sup>7</sup>. Unfortunately this statue has been lost since the Second World War<sup>8</sup> so that no modern evaluation could be performed. Votive offerings were grateful gifts to the god Aesculapius from the patient. They typically represented healthy or cured body parts, often as



carved tablets or sculptures. As this statue clearly depicts a pathologic state, a more attractive hypothesis is that it forms part of a known collection of similar statues

illustrating various diseases. These statues were manufactured in Smyrna on the Ionian coast during the Hellenistic period<sup>9</sup>. They were clearly not votive offerings, but instead were part of a repertoire of didactic material dedicated to diagnostic training for the scholars of the local Hippocratic medical school<sup>10</sup>. Our statue may have been an early three-dimensional teaching aid!

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## Scum summary

SIR — In response to our Scientific Correspondence<sup>1</sup>, Lewin speculated<sup>2</sup> that the main components of tea scum are epidermal waxy lipids removed from the tea leaves by boiling water. This hypothesis does not explain why no scum forms when tea leaf is infused with hot distilled water, distilled water containing calcium chloride or soft water — or why tea scum always forms in temporary hard water, even when instant tea, which contains no insoluble waxy components, is used.

Jones<sup>3</sup> and Enzweiler and de Oliveira<sup>4</sup>, on the other hand, believe the brown film on tea is formed by the physical<sup>3</sup> adsorption of coloured tannin on calcium carbonate which (we confirm) forms on the surface of hot temporary hard water. Although this hypothesis is nearer the mark, it does not explain why the formation of the dark film is greatly dependent on the oxygen content of the atmosphere above the tea brew<sup>1,5</sup>; or why the tea scums we have analysed<sup>6</sup> contain 3–7 wt % calcium and 24–40 wt % carbon whereas the composition expected from CaCO<sub>3</sub> plus an adsorbed layer of tea polyphenolics would be just under 40 wt % Ca and just over 12 wt % C.

It is clear from the above evidence, together with other experimental findings<sup>5,6</sup>, that tea scum consists mainly of insoluble oxidized tea polyphenolics together with calcium carbonate.

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# Mercury pollution from deforestation

SIR — High mercury levels in the blood of fish-eating people in the Amazon have been attributed to gold mining activities conducted by informal miners. However, the high deforestation rate in the region has not been recognized as contributing to this environmental problem. About 90 tonnes of organic mercury from the biomass are estimated to be emitted annually to the atmosphere and precipitated in the aquatic systems for rapid transformation into methylated forms. This is a conservative assessment and may be more than six times this rate.

Mercury emissions from informal mining operations, or *garimpos*, have been identified as a potential epidemic in the Amazon region. These operations expanded in Brazil in the 1980s as a consequence of poorly conducted agricultural policies, increased inflation and high unemployment. Mercury is used to amalgamate gold, and a misunderstanding of adequate technical procedures and side-effects has caused considerable occupational hazards and contamination of other communities not directly involved with mining activities<sup>1</sup>.

Based on gold production, one estimate has been made that 170 tonnes of Hg enter *garimpos* annually<sup>2</sup>. Mercury losses of 40% are typical when amalgam is distilled in open pans, so about 70 tonnes of Hg are lost if retorts are not used at all by miners. About 80% of these losses are emitted directly to the atmosphere, while the remainder is discharged with amalgamation tailings to watercourses<sup>3</sup>. Toxic vapour taken up in workers' lungs is mirrored by high Hg content in their urine. Samples as high as 840 p.p.b. Hg have been recorded (normal levels are below 10 p.p.b.)<sup>3</sup>.

High Hg levels have been observed in fish from areas well away from mining activity, and Hg has been measured in blood samples from residents of Jacareacanga, a city 200 km south of mining activity on the Tapajós River, and from inhabitants of a fishing village, Brasília Legal, 250 km to the north. Fish is the

ESTIMATED Hg EMISSIONS FROM THE AMAZONIAN BIOMASS DURING DEFORESTATION

| Biomass             | Average (tonnes per ha) | Hg (p.p.m.) | Hg release efficiency (%) | Hg released (g per ha) |
|---------------------|-------------------------|-------------|---------------------------|------------------------|
| Above-ground wood   | 260                     | 0.05        | 90                        | 11.7                   |
| Above-ground leaves | 9                       | 0.05        | 90                        | 0.4                    |
| Above-ground roots  | 20                      | 0.05        | 90                        | 0.9                    |
| Below-ground roots  | 35                      | 0.05        | 20                        | 0.4                    |
| Fallen trunks       | 16                      | 0.05        | 90                        | 0.7                    |
| Humus               | 11                      | 0.3         | 20                        | 0.7                    |
| Soil organic matter | 47                      | 0.3         | 20                        | 2.8                    |
| Total               | 398                     |             |                           | 17.6                   |

main diet in these communities, and about 90% of the Hg in fish is methylated<sup>4</sup>.

Mercury has an affinity for organics in soil; raw humus ranges from 0.2 to 1.9 p.p.m. Hg<sup>5</sup>. Our analysis of 0.8 p.p.m. Hg in fulvic acid isolated from a non-mineralized and uncontaminated soil from British Columbia confirms the capacity of these substances to take up Hg from any source. These complexes will methylate by biotic or abiotic processes to be channelled into the food chain<sup>6</sup>.

In 1972, fish from reservoirs in northern Manitoba had high levels of Hg. No man-made source could be identified precisely, but the high Hg background of organic soils associated with a new impoundment suggested that biomethylation had occurred. Natural forest fires were also attributed as a source, but the amount released annually was calculated at 20 g ha<sup>-1</sup> (less than 0.02% of provincial annual natural emissions)<sup>7</sup>, creating a high short-term emission pulse. The evaluation assumed 0.4 p.p.m. as the Hg level in timber, but only 0.08 p.p.m. was thought to be lost during fires.

Worldwide, wild forest fires are estimated to release 20 tonnes Hg to the atmosphere, less than 1% of natural emissions<sup>8</sup>. Intentional wood combustion represents 60–300 tonnes Hg (about 5% of man-made emissions in 1983)<sup>9</sup>, an estimate based on 0.1–0.5 p.p.m. Hg in wood. Today, given the high rate of deforestation by fires in the Amazon, Hg emissions derived from wood combustion must be a more important source. Deforestation in the Amazon is between 35,000 (1987) and 50,000 (1988) km<sup>2</sup> per year, producing mostly cattle pasture<sup>10</sup>.

Natural Hg levels in plants range from 0.001 to 0.1 p.p.m. (dry weight). In forest ecosystems, this increases to 0.01–0.3

p.p.m.<sup>11</sup>, whereas crops grown in soils containing less than 0.04 p.p.m. Hg vary from 0.004 to 0.09 p.p.m.<sup>12</sup>. Little is known about Hg distribution in the Amazon flora but aquatic macrophytes show levels between 0.1 and 1.0 p.p.m.

The temperature range encountered in vegetation fires is between 650 and 1,100 °C (ref. 13). At 200–300 °C, destructive distillation of about 85% of organics occurs. Mercury compounds are volatile between 25 and 450 °C, and organic mercurials usually have lower boiling points than inorganic ones. When fossil fuel is burnt, more than 90% Hg is lost<sup>14</sup>. Because most Hg in wood is present in organic form, it can be assumed about 90% of Hg is lost from above-ground biomass. It is also reasonable to infer that, as with other metals, the remainder becomes weakly bound to the ash to be leached by runoff water.

We have calculated the amount of Hg emitted by deforestation from estimates of biomass distribution in the Amazon<sup>15</sup>. We assume most Hg compounds are released from above-ground biomass even without complete combustion, whereas only a minor amount volatilizes from surface soil. Our evaluation of the efficiency of Hg release is shown in the table. Using a conservative estimate of Hg levels in plants and organic matter of 0.05 and 0.3 p.p.m. respectively, the unit Hg emission is 17.6 g ha<sup>-1</sup> (1.76 kg km<sup>-2</sup>). Considering that 50,000 km<sup>2</sup> was burnt in 1988, 88 tonnes of Hg were probably emitted to the atmosphere that year, (26% above the estimate for mining emissions). The total area consumed up to 1991 is estimated at 404,000 km<sup>2</sup> (ref. 16). So more than 710 tonnes Hg have been released from this source. If the wood levels claimed by Nriagu<sup>9</sup> are used (0.1–0.5 p.p.m.), Hg emissions from above-ground wood alone would be 117–585 tonnes per year.

Release of water-soluble Hg[II] from burning fossil fuel depends on the presence of chloride and/or active particulate matter but can be as high as 50% of total mercury<sup>14,17</sup>. Combustion gases from forest fires would follow the same pattern, representing imminent danger as methylation is enhanced in aquatic environments. In contrast, the conditions during amalgam-burning do not favour oxidation of mercury vapour either thermodynamically

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cally or kinetically<sup>17</sup>. Deforestation has not been considered in monitoring programmes in the Amazon, although fly-ash transport is now being investigated. It is clear that deforestation is a major source of mercury emission in a form more dangerous than that emitted by *garimpos*. Mercury emissions from any source must be stopped but all significant villains should be recognised.

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## CO<sub>2</sub> and diatom mats

**SIR** — Levels of atmospheric CO<sub>2</sub> are thought to have been substantially higher during much of geological history than at present<sup>1</sup>. It has been suggested that the decrease in stromatolites in the Phanerozoic fossil record could partly be explained by declining levels of atmospheric CO<sub>2</sub>, leading to CO<sub>2</sub> limitation<sup>2,3</sup>. Here I sug-

gest that CO<sub>2</sub> limitation was also a contributing factor to the absence from the fossil record for the past 4.4 million years of the previously vast laminated diatom assemblages of the eastern equatorial Pacific<sup>4</sup>.

To enter the fossil record, biological material must be produced, exported and preserved. The export production of, for example, rhizosolenid diatom assemblages, is controlled by grazing, buoyancy, cell size and aggregation into mats<sup>5</sup>. The preservation of laminated sediments depends on low oxygen in the water to prevent a benthic community from causing sediment bioturbation, although in the case of the Neogene mats the amount of diatom material apparently overwhelmed the benthos<sup>4</sup>. Growth rates in phytoplankton are mainly controlled by primary production, and it is this factor that is influenced by levels of inorganic carbon. There must also be a link between growth rates and export production, because growth rates must be greater than loss if a mat is to form, algal concentration can affect grazing and aggregation, and growth rate can directly affect cell size.

Recent laboratory work on marine planktonic diatoms, at least one of which, *Rhizosolenia alata*, forms blooms<sup>6</sup>, demonstrates that, given nutrient conditions typical of the onset of a high-latitude spring bloom, cell-doubling rates are correlated with concentrations of dissolved gaseous CO<sub>2</sub> (ref. 6). In this experiment, CO<sub>2</sub> concentrations were kept constant for 5–6 days. Cell doubling decreased dramatically at levels below ambient, but did not increase more than about 10% at free CO<sub>2</sub> concentrations over ambient. However, during intense growth in nature, such as in a bloom, free pCO<sub>2</sub> can become severely depleted<sup>7</sup>. Such depletion, caused by photosynthetic uptake and the resulting rise in pH of the surface water, is considered to be one cause for the collapse of spring diatom blooms<sup>8</sup>. Concentrations of CO<sub>2</sub> in sea water are dependent on dissolved inorganic carbon and pH, which controls the equilibrium concentrations of the species of inorganic carbon. Bulk inorganic carbon concentrations are a function of physical factors such as levels of atmospheric CO<sub>2</sub>, temperature, surface mixing and salinity<sup>9</sup>. Biological factors such as the uptake and remineralization of inorganic carbon also affect oceanic inorganic carbon and pH (ref. 10).

Atmospheric concentrations of CO<sub>2</sub> 21 million years ago are estimated to have been about 4.5 times their present levels, suggesting that levels of dissolved inorganic carbon were higher<sup>10</sup>. Surface sea water in the equatorial Pacific near Micronesia was pH 7.4, for example, in contrast to present-day values of about 8.2 (ref. 11). At 34 ‰ salinity, 18.5 °C and pH 7.4, about six times more dissolved inorganic carbon exists as free CO<sub>2</sub>, than at pH 8.2, or roughly 4.2 versus 0.7% free CO<sub>2</sub> (see figure). Given sufficient quantities of other nutrients, diatoms would have shown elevated doubling rates at

higher cell concentrations than would be possible today, resulting in more extensive populations. Conversely, as atmospheric levels of CO<sub>2</sub> decreased and the pH of surface sea water increased to near present-day levels, free CO<sub>2</sub> in surface waters decreased. As a result, diatom populations became CO<sub>2</sub>-limited, leading to a decrease in diatom growth rates. The CO<sub>2</sub> limitation on diatom growth contributed to the disappearance from the fossil record of the Neogene diatom assemblages.

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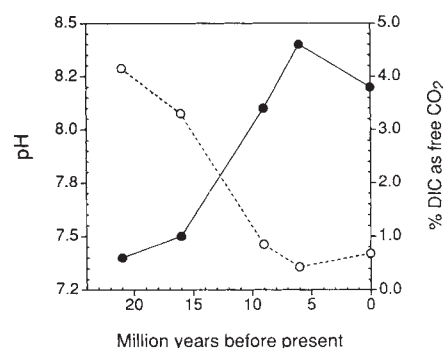
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## Mitochondrial DNA inheritance

**SIR** — Although the inheritance of mitochondrial DNA (mtDNA) was thought to be maternal, a low level of paternal transmission does occur in some animals<sup>1,2</sup> and plants<sup>3</sup>. In the marine mussel *Mytilus*, the results of laboratory crosses suggest extensive paternal transmission of mtDNA genotypes, as well as maternal inheritance<sup>4</sup>. This explains why many mussels from diverse geographic locations can possess two mtDNA genomes which show high nucleotide sequence divergence (10–20%)<sup>5,6</sup>. We have obtained evidence that whereas female mussels have only one mtDNA genome that is transmitted to eggs, male mussels have, in addition, a second genome that is transmitted preferentially to sperm.

Cloned DNA from the two genomes in *Mytilus edulis* (*F* and *M*<sup>4,5</sup>) has been used to probe genomic DNA prepared from somatic tissue and gametes (*a* in fig. 1). Males consistently give strong signals for both genomes in somatic tissue, but for only the *M* genome in sperm. Females consistently give a strong signal for the *F*, but not for the *M* genome, in both somatic tissue and eggs. These sex and tissue specific differences are mirrored when



Sea surface CO<sub>2</sub> (open circles) and pH (filled circles) changes during the past 21 million years. Values for pH from ref. 11; free CO<sub>2</sub> values calculated from the dissociation constants for dissolved inorganic carbon (DIC) given in ref. 12.

gest that CO<sub>2</sub> limitation was also a contributing factor to the absence from the fossil record for the past 4.4 million years of the previously vast laminated diatom assemblages of the eastern equatorial Pacific<sup>4</sup>.

To enter the fossil record, biological material must be produced, exported and preserved. The export production of, for example, rhizosolenid diatom assemblages, is controlled by grazing, buoyancy, cell size and aggregation into mats<sup>5</sup>. The preservation of laminated sediments depends on low oxygen in the water to prevent a benthic community from causing sediment bioturbation, although in the case of the Neogene mats the amount of diatom material apparently overwhelmed

### Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.



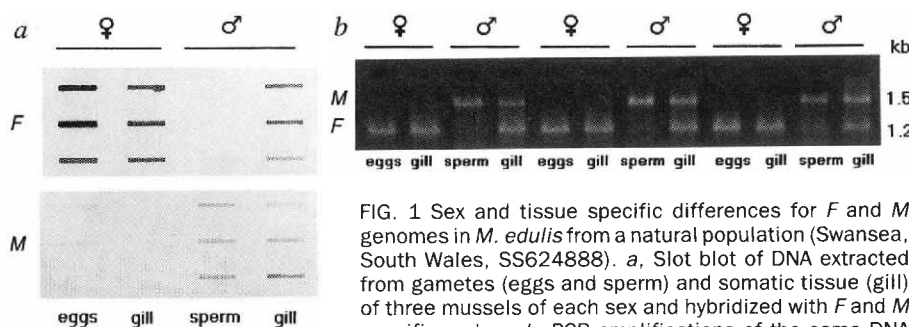


FIG. 1 Sex and tissue specific differences for *F* and *M* genomes in *M. edulis* from a natural population (Swansea, South Wales, SS624888). *a*, Slot blot of DNA extracted from gametes (eggs and sperm) and somatic tissue (gill) of three mussels of each sex and hybridized with *F* and *M* specific probes. *b*, PCR amplifications of the same DNA using primers specific for the *F* and *M* genomes. The separate *F* and *M* amplifications for each DNA sample were loaded in the same well of an agarose gel to facilitate comparison. The results shown are representative of a study in which gametes and somatic tissue were examined from 16 females and 17 males. Note the weak *F* signal obtained with sperm DNA for the male at the right of the panel. Further details are available from D.O.F.S. on request.

using genome specific PCR primers (*b* in fig. 1). Moreover, in females, the *M* genome is undetectable using PCR.

If females lack the *M* genome, males will inherit it from their fathers. If the *M* genome is partitioned preferentially into sperm, both sexes will inherit the *F* genome from their mothers. It can be concluded that the two genomes have separate transmission routes. Some males give a weak *F* signal for sperm (*b* in fig. 1), suggesting that the paternal transmission route might be leaky.

Separate maternal and paternal transmission routes could allow long-term maintenance of both mtDNA genomes within mussel populations in the face of genetic drift. This might explain the evolution of the high level of divergence between the genomes. In male zygotes, copies of the *F* genome derived from the egg will vastly outnumber the few copies of the *M* genome derived from sperm. It follows that in males, the *M* genome must replicate faster than the *F* genome to be detectable at a high level in blotting experiments. This implicates interactions between mtDNA and sex-determining factors.

Nuclear modifiers causing uniparental inheritance of cytoplasm might have been selected in evolution to prevent the rapid spread of selfish and harmful cytoplasmic particles to all lineages within species<sup>7-9</sup>. The maternal inheritance of mtDNA in most organisms could be accounted for in this way. *M. edulis* presents no contradiction. Although the inheritance of mtDNA is biparental, individual mtDNA genomes are inherited uniparentally through either the female or male line of descent. Thus,

mussel populations might be safeguarded against the rapid spread of selfish mtDNA genomes by the restriction of such genomes to either male or female clones. The level of protection should depend on the amount of leakage.

The analysis of different tissues within individual animals is not usual in studies of mtDNA variation. Thus separate maternal and paternal routes of mtDNA

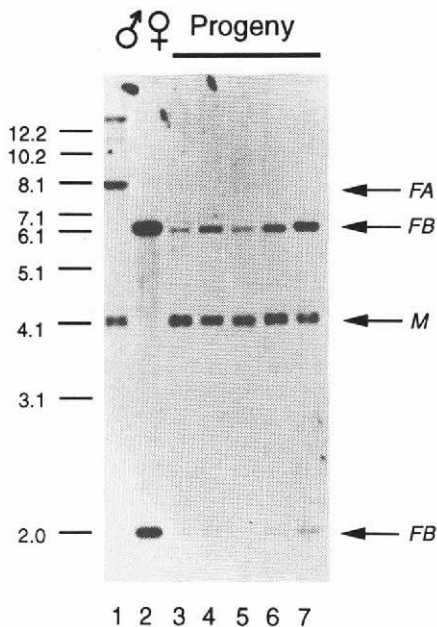


FIG. 2 DNA from the father (lane 1), mother (lane 2) and five sons (lanes 3 to 7) of family No. 16 (ref. 2) was restricted with *EcoRI* and probed with a mixture of clones that hybridize to 6-kb and 2-kb bands of the *FB* profile, the two 4-kb bands of the *M* profile and one of the two 8-kb bands of the *FA* profile (original descriptions of these profiles in refs 2, 5). The mother had the typical *FB* profile and the father was heteroplasmic having inherited the *M* type from the father (the *M* type does not occur in females<sup>2,5</sup>) and the *FA* type from the mother. All sons inherited the mother's mtDNA diagnostic bands (6 and 2 kb). No son had the diagnostic band of the father's maternal mtDNA (8 kb), but all had the diagnostic band of father's paternal mtDNA (4 kb). Bands in the male parent above 8 kb result from incomplete digestion.

inheritance might occur widely, but have been missed in other organisms.

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**SIR** — The exceptionally high rate of biparental inheritance in the blue mussel *Mytilus*<sup>1,2</sup> appears to defy the view that uniparental transmission of organelle DNA evolved to prevent the spread of selfish deleterious mutations of cytoplasmic DNA throughout the species<sup>3,4</sup>. We have now grown progeny from the crosses in which we have demonstrated biparental inheritance<sup>2</sup> to the age they could be sexed and found that biparental inheritance was limited to sons.

In two crosses, the father's paternal and maternal mtDNA could be distinguished from each other and from the mother's mtDNA. In these crosses we found that male progeny inherited from the father only his paternal mtDNA (see fig. 2). Thus, in *Mytilus* mtDNA transmission is 'doubly uniparental', with non-anastomosing female and male lineages.

Deleterious mutations arising in a male's maternal mtDNA will not be passed to offspring, and mutations arising in a male's paternal mtDNA will affect only his sons and filial grandsons. Mutations arising in a female's mtDNA will affect her sons and daughters, but will be transmitted only through her daughters. In all cases mutations will be confined within the lineages they arose and will be removed from the population with the extinction of these lineages. Far from defying the theory of evolution of uniparental transmission of organelle DNA, mtDNA transmission in *Mytilus* provides a much needed empirical support for the theory.

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# Managing Prometheus

John Mulvey

**Prometheus Bound: Science in a Dynamic Steady State.** By John Ziman. Cambridge University Press: 1994. Pp. 289. £16.95, \$24.95.

PROMETHEUS, who displeased the Gods by bringing the secret of fire to Earth, has seldom been more active. The frontiers of human understanding have expanded further and more rapidly in this century than in any earlier period, while the applications of modern science and technology are dramatically transforming the ways in which we live and work, and treat disease. As the possibilities grow almost explosively, the aspirations of scientists and engineers wishing to follow new opportunities for discovery, and the expectations of society looking for the benefits, rise to levels threatening to exceed the resources available.

The theme of John Ziman's thesis is that Prometheus has exhausted his lines of supply: he is now effectively on a tight leash. All the present trials and tribulations of the scientists — and here he really means academic scientists and engineers — stem from the abrupt need to adjust to level, or almost level, funding for research rather than continuing to enjoy the doubling of resources every 15 or so years, a trend that was typical before the 1970s.

It must be a truism that having through their efforts in the pursuit of knowledge wrought such manifestly great changes in the nature of society, scientists cannot expect to remain immune to new stresses. Much has been happening to bring radical change to university life, and inadequate funding has been accompanied by additional demands. Ziman does not, for example, consider structural changes in the United Kingdom such as the doubling of the number of universities, the projected doubling of the number of students entering higher education with no corresponding increase in staff, and the uniquely British reduction in government finance for research in other sectors that increases the pressure on the science base.

The UK government's 1993 White Paper (policy document) on science and technology policy, the first for more than 20 years, stresses the role of the science base in wealth creation. The emphasis on "management" of the research base and particularly the proposition that this is best overseen by industry — as the end-users of the 'product' — is for many scientists a worrying development. Gradually but surely, the weight of support may, it is feared, move away from research determined by scientific criteria towards providing a short-term, problem-solving service. Instead of Prometheus, whose name

means 'forethought', we risk being led by his brother Epimetheus.

For those inside academic science, the past decade has certainly been "dynamic" — indeed, reminiscent of suffering under the ancient Chinese curse: "May you live in interesting times". Ziman comments that, faced with a situation in which the 'goal posts' seem to be continuously moving, "the responsible authorities at every level have been improvising wildly". This is all very far from a "steady state", the phrase he uses, rather misleadingly given the context, to characterize level funding — a situation he regards as permanent, indeed axiomatic. Others will take issue with this position, arguing that an investment so fundamental to growth in the economy should at least keep pace with that growth. This would not satisfy all the pressure from scientists, but would better enable change to be managed and allow adaptation to new challenges and demands with less of the damaging waste and turmoil typical of recent years.

Writing from the vantage point of a former departmental head of physics at

the University of Bristol, who then spent six years as director of the Science Policy Support Group until retiring at the end of 1992, Ziman succeeds very well in his purpose of describing the structures of academic science and the motivations of scientists driven by the intense competition of research at the frontiers. He gives an excellent analysis of the many dangers that threaten the future success of the enterprise and are the causes of much anguish in the laboratories.

In the midst of radical change it is essential to ensure that those aspects of past practice that have been the secrets of success are retained. Ziman offers a translation of the new buzz-words of management, eloquently demolishing the fallacies and warning of the dangers of clumsy, uncomprehending application. He deplores the way "selectivity" frequently leads to "arbitrary decisions to close down small, but beautiful, research operations [and] stifle novel developments". Academic freedom must be protected, its essence being "to leave openings for untypical people with untypical talents".

Although cast in a UK context, Ziman's analysis will strike echoes everywhere. This is a book for all who wish to understand the concerns of those working at the frontiers of science to set Prometheus free. □

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"THE Twelve Apostles", one of Australia's most famous coastal localities, situated in western Victoria. The stacks of Tertiary-age limestone rise up to 46 metres above the sea surface. This photograph is taken from the cover of *The Evolving Coast* by Richard A. Davis, a gloriously illustrated volume in the Scientific American Library series that introduces the general reader to the "science behind the natural drama of coasts". Scientific American Library/W. H. Freeman, £19.95.



## Silent travellers

Robert Desowitz

**Power Unseen: How Microbes Rule the World.** By Bernard Dixon. W. H. Freeman: 1994. Pp. 237. £16.99, \$22.95.

**The Outer Reaches of Life.** By John Postgate. Cambridge University Press: 1994. Pp. 276. £16.95, \$22.95.

SIXTY-seven years have passed since Paul De Kruif introduced the microbe to an anxious, largely defenceless public. Although the AIDS virus has spawned a flourishing industry in the arts and letters, microbes have, since then, largely withered on the literary vine. And, come to think of it, in De Kruif's *Microbe Hunters* it was the microbiologist, not the microbe, who had star billing.

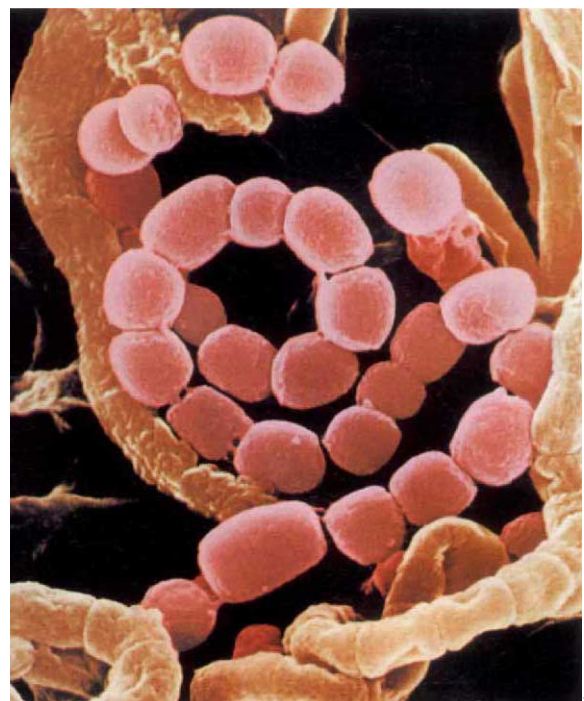
These two popular science books make a significant new contribution in educating the public in the ways of microbes. They differ in content and style, reflecting the occupation and interests of each author. Bernard Dixon is a scientist who has become a science writer; a former editor of *New Scientist*, he has written seven trade books and has a monthly "Microbe of the Month" column in the *Independent*. John Postgate, a fellow of the Royal Society and a distinguished microbiologist, is a scientist who writes. Dixon 'talks story', as they say here in Hawaii; Postgate-the-professor-emeritus treats members of the reading public as if they were his students — he teaches.

The manner in which the two authors discuss the nitrifying bacteria is an example of their divergent interests. Dixon

focuses on the nitrifiers' destruction of stone monuments and provides only a cursory two-sentence description of the chemical mechanism of their acid-forming properties. Postgate, in considering how a microbe can live off an insoluble substance, focuses on its chemical physiology and gives the reader a chemistry lesson in enzymatic action and acid end-products. Another difference: Dixon calls *Nature* a "journal", Postgate calls it a "magazine".

*Power Unseen* covers the entire microbial gamut in 75 short essays. But the gamut is markedly skewed towards the pathogenic bacteria. Viruses get short shrift, seven essays (eight if you count the bacteriophage). It's all very even-handed; the AIDS virus gets the same three-page treatment as *Micrococcus sedentarius*, the bacterium accused of causing the effluvium rising from athletes' toes and socks. Dixon forgoes the opportunity to lead the reader from the AIDS virus to the phenomenon of the opportunistic disease caused by otherwise benign, quiescent microbes, particularly the protozoa. *Cryptosporidium*, *Toxoplasma* and *Pneumocystis* (although probably not a protozoan) are not mentioned. The only protozoan represented is the malaria parasite, *Plasmodium*, and its three-page allotment is restricted to a not entirely perceptive account of its pyrogenic potential. A pathogen responsible for more than two million deaths a year deserves better treatment — in all sorts of ways.

Dixon relishes the telling of cascade stories that stretch our imagination and, occasionally, our credulity. David Lloyd George is minister of munitions. He needs acetone to make cordite. He turns for help to a Jewish East European immigrant bacteriologist, Chaim Weizmann. Weizmann discovers that *Clostridium acetobutylicum* produces acetone as a metabolic end-product. The war is won and Lloyd George is prime minister. Arthur Balfour is his foreign secretary. Weizmann asks for, and is rewarded with, a Jewish homeland. *Clostridium*



Jeremy Burgess/SPL

Spore chain of the soil bacterium *Streptomyces lividans*.

*acetobutylicum* has fathered Israel.

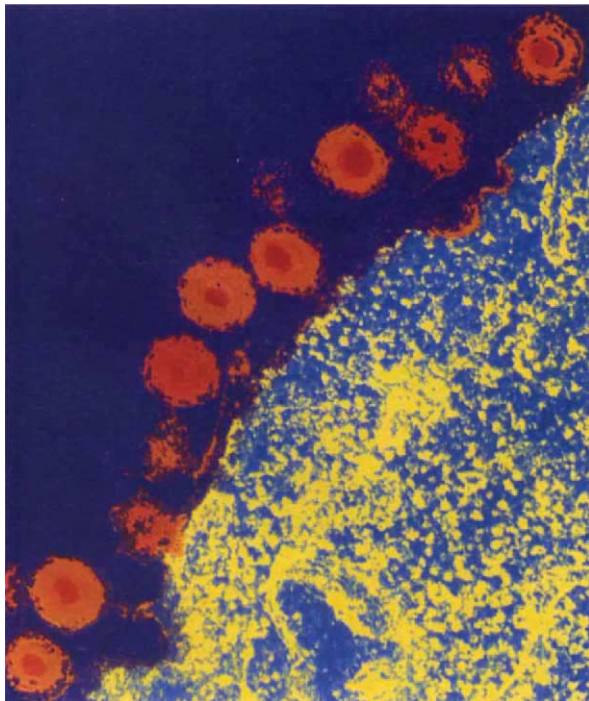
*The Outer Reaches of Life* eschews the pathogen and directs attention to those microbes that are, in anthropomorphic judgement, the wretched of the Earth. These are the 'simple' organisms that have made the not-so-simple adjustments to environments that are the 'outer reaches of life' — the cold of Antarctica, the near-boiling water of hot springs, the near-nutritionless benthic depths, the corrosive highly acidic and alkaline media. Postgate speculates that if life can evolve and be sustained in the harshest conditions of Earth, then creation and complex evolution is possible on the other planets of our Solar System.

Explaining the mechanisms that allow microbial life in these specialized, restricted environments involves a lot of chemistry. Postgate is not entirely successful in his efforts and it can be pretty heavy going for the nonscientific audience to whom the book is directed. But the reader who perseveres will be rewarded with a broadened vision of the nonpathogenic bacteria and the ways in which they have insinuated themselves into seemingly unsustainable habitats.

I recommend both books; Dixon's for a good read, Postgate's for solid popular science writing in a neglected field of microbiology. Individually or together they are not the new *Microbe Hunters*, but they are informative and, especially in the case of *Power Unseen*, entertaining. □

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A. B. Dowsett/SPL



Human herpes virus type 6 on the surface of an infected cell.



# The cub and the pope

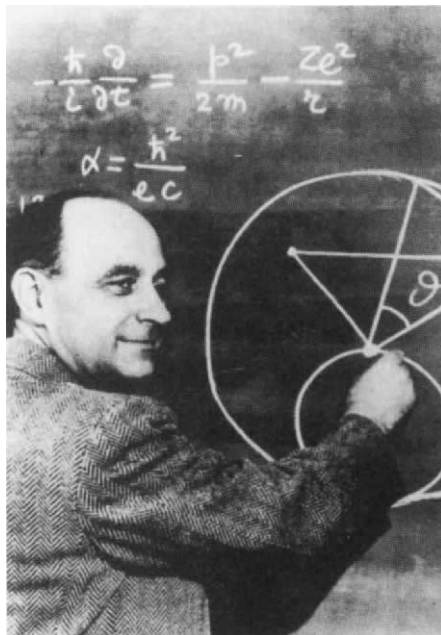
G. F. Bignami

**Enrico Fermi: Ricordi di allievi e amici.** By Bruno Pontecorvo. Edizioni Studio Tesi, Pordenone, Italy: 1993. Pp. 214. L30,000.

BRUNO Pontecorvo died in Dubna on 24 September 1993 and did not have a chance to see in print his last work: the Italian (and only non-Russian) version of *Enrico Fermi v vospominaniyakh uchenikov i druzej*, which he had written with V. Polrovskij back in 1972. He did, however, read the final proofs, and put in meaningful corrections in his shaky but lucid handwriting. A new, vivid portrait of Enrico Fermi comes out of the Pontecorvo stories, which make up most of the book. Other parts are less original. Some, such as memories of Emilio Segrè and others, have gone through a double translation cycle, from English to Russian to Italian, and have lost some of their freshness in the process. One generally feels that the Russian-Italian translation has received more attention (possibly by Bruno himself) than the English-Russian-Italian sequence, which also features a few funny mistakes. (On page 159 we discover a reference to "Adamar", obviously a transliteration from the economical Russian spelling of the name of the Franco-American mathematician Hadamard).

The Pontecorvo testimony is of unique human and historical value for the period of the roaring thirties in nuclear and atomic physics, when Bruno worked with Enrico at via Panisperna in Rome. In the hierarchy of that famous group, Fermi was "the pope", infallible in matters of physics, and Pontecorvo was "the cub", the youngest and last to join. For this, he had to pass the customary entrance exam under the ice-cold eyes of the pope. Afterwards, Fermi took the time to explain to him that "today, theoretical physicists are like Aegyptologists: if they are not absolutely first class and produce unique, outstanding work, they are of no use and have, obviously, taken up the wrong profession. Experimentalists, on the other hand, can produce useful work even if they are just average: they can, for example, accurately measure the density of all elements. This would be very useful work." Pontecorvo, later to become a very famous theoretician himself, was of course asked to join the group, but as an experimentalist. (He took his revenge at tennis, however. Bruno was throughout his life an excellent, tournament-class player, and, 12 years Fermi's junior, would consistently beat Fermi to pulp, judging him only an average player.)

Fermi had a quiet, inner consciousness of his natural gifts and of his immense culture in physics, and this gave him the self-confidence required for producing truly original work. We learn that he acquired this self-confidence, still lacking in his greener years, after a visit to Paul Ehrenfest, in Leiden, for three months in 1924. He was then 23 and knew, and remembered perfectly, all that could be learned about physics at the time, but needed the reassurance of an accepted leader in the field. Fermi remained forever grateful to Ehrenfest, whom he considered to have had an enormous influence



Enrico Fermi (1901–54) had a "quiet, inner consciousness of his natural gifts and of his immense culture in physics, and this gave him the self-confidence required for producing truly original work".

on the development of physics in Europe. The other person whom Fermi, in his Italian years, thought at least his equal in theoretical physics was Ettore Majorana, the "great Inquisitor" in the via Panisperna, religion-inspired nomenclature. Majorana was in fact the only one who could make the pope ill at ease, with the acuity of his remarks, sometimes biting harsh, but always right. The pope, let us not forget, was a very young one: a chair in theoretical physics at 25, Accademico d'Italia at 27, of course the youngest ever. He did not especially boast about his position, but he liked the magnificent uniform that went with the title and did not mind spending 7,000 lire on it, more than three months' his professor's salary.

Pontecorvo, from a much wealthier upper-class family and with a definite suspicion of snobbery, was at the time living at the Young Men's Christian Association in Rome, and walking to via Panisperna daily, to record in his diaries the making of physics history. As a result, the

book now contains both anecdotes and profound scientific and human analysis of the work of Fermi and the group. All is invaluable material for the understanding of a period from which very few witnesses now remain. We know that Fermi named the "neutrino" (the small neutral one) the hypothetical Pauli particle; less well known is that he did so in October 1931, well before the announcement of the discovery of the "neutrone" (the big neutral one). This great moment in science, incidentally, received the usual blasé response from Majorana: "at last, Chadwick has seen the neutral proton", while most of the world was puzzled by the reported results. Shortly afterwards, Fermi wrote his famous theory about beta-decay, sent it to *Nature* and had it rejected as "too abstract, not interesting to our readership". (The work, a classic, appeared in *La Ricerca Scientifica*, the CNR journal.)

A strong new impression of Fermi jumps out of Pontecorvo's pages. It is impossible to draw a line between the man and the scientist: he would teach not only physics, but also how to understand its spirit and how to fall in love with it. In the making of a work of science, he was conscious of the high moral responsibility in publishing it, and was attentive to the accuracy of every word. Above all, a vast dose of common sense: no need to look for the "ultranuovo" when existing laws are perfectly adequate — provided, of course, that you know them well enough.

On the period that Fermi and Pontecorvo spent together in Italy, and more, another recent book is especially well researched and written: Miriam Mafai's *Il Lungo Freddo* (Mondadori, 1992), the story of Pontecorvo's life, including, of course, the period after his disappearance to the Soviet Union in 1950. Comparing it with Pontecorvo's own writing, actually preceding Mafai's book but independent, is illuminating, especially on the image that Pontecorvo had of Fermi, a dominant figure in his mind even after many years of geographical separation. □

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■ Fermi and Pontecorvo are among several Western scientists accused in a new book as acting as spies to give Moscow atomic secrets in the 1940s. In *Special Tasks: The Memoirs of an Unwanted Witness — a Soviet Spymaster*, Pavel Sudoplatov, an intelligence chief during the Stalin era, claims that Oppenheimer, Fermi, Szilard, Gamow and Pontecorvo provided, or knowingly allowed the transfer of, scientific information essential to the Soviet atom bomb project. The book was published last week by Little, Brown at £18.99 and will be reviewed in these pages on a later date. See also News in this week's issue.



# Thinking ahead

Martin Shubik

**Game Theory and Strategy.** By Philip D. Straffin Jr. *Mathematical Association of America*: 1994. Pp. 244. \$27.50 (pbk).

**Theory of Moves.** By Steven J. Brams. *Cambridge University Press*: 1994. Pp. 248. £36, \$54.95 (hbk); £13.95, \$17.95 (pbk).

*GAME Theory and Strategy* is an elegant, crystal-clear expository work. Philip Straffin presents the key ideas behind finite games in strategic and coalitional form, and provides many simple and intuitively appealing examples of applications to business, politics, economics, social psychology, philosophy and evolutionary biology. Key concepts are emphasized and clearly explained. Here is a book for interested lay people, undergraduates or graduates with little knowledge of mathematics; even high-school seniors might appreciate it.

Virtually all the basic ideas discussed were published between 1944 and 1974. The curious general reader may wish to learn what has happened more recently. There have been several important new developments that are, for the most part, elaborations of the noncooperative equilibrium concept. They include the concept of perfect equilibrium, the development of game theory with incomplete knowledge of the rules of the game and the treatment of lack of common knowledge. But these topics require a depth of mathematical explanation inappropriate for Straffin's purposes.

Game theory has matured since its creation by John von Neumann and Oskar Morgenstern in 1944 and is now a respected field of study. The latest challenge is the development of new basic ideas. This is especially true for dynamics. Except for two-person zero-sum games there is no generally satisfactory dynamic game theory. Most games and actual strategic problems are neither purely cooperative nor noncooperative and we still lack a clearly correct way to handle them. This is not to criticize Straffin's fine book, but merely to point out that game theory has some way to go and that future directions and problems cannot be inferred from his otherwise thorough account.

Such issues are, however, the concern of Steven Brams, an imaginative and innovative scholar who has done much to promote the use of game-theoretic thought in political science. In *Theory of Moves* he presents an ambitious new approach to some central problems in modelling games for the study of the dynamics of conflict and cooperation. Unfortunately his attempt, like those of

T. C. Schelling and N. Howard before him, identifies many of the problems but fails to offer any satisfactory solutions.

He begins by claiming that there are several features of his 'theory of moves' that make it superior to the classical theory of games. Game theory, he says, has neglected the conditions under which players move in a specified order; players, he reminds us, think ahead not just to the immediate consequences of making moves, but also to the consequences of counter-moves to these moves, counter-counter-moves and so on. To illustrate some of the problems, he spends several pages discussing the 'truel', the name for a three-person version of a duel that I coined in 1954 when asking: "Does the fittest necessarily survive?"

After corresponding with the ethologist Konrad Lorenz, investigating with graduate students games involving more than three players, and contemplating the truel of the Russian and British empires with Afghanistan, I concluded that just about anything goes: that is, the order of moves and likely outcomes are highly dependent on players and context. The standard formal methods of extensive form game theory are adequate for describing many games where the order of moves is part of the strategy, but rather poor for describing 'free-form war gaming' in which conversation and discussion are involved. This point was already implicit in Schelling's work.

Brams's second example is a three-by-three bi-matrix game originally used by L. S. Shapley in 1964 to reveal a basic weakness in blindly accepting as a solution the Nash noncooperative mixed-strategy equilibrium. Shapley noted that the alternating best-response six-cycle does better than the mixed strategy. Brams's conclusion confirms Shapley's view; but at the level of formal theory it does not, to my mind, go beyond Shapley's work.

Brams's central point is that threats, inferences about threats and the plausibility of threats are important. But to a great extent this is what Schelling said in 1960 in *The Strategy of Conflict* (Harvard University Press). At the time, I wrote a fairly unfavourable review of this book. In retrospect, I should have been more complimentary. I failed to appreciate Schelling's skill and perception in pointing out a host of problems in modelling international gamesmanship and brinkmanship, problems not easily solved by conventional game-theoretic methods. All I saw was his lack of clear understanding of many of the features of formal game theory. Although Brams clearly demonstrates that he knows far more formal game theory than Schelling, I fail to find where his work represents either a significant extension of Schelling's analysis or a solid contribution to formal game theory.

Brams's book may be of use to readers who are ignorant of formal game theory but familiar with conversational game theory using two-by-two matrices, usually based on the Prisoner's Dilemma. But it will be of little benefit to people who know some elementary formal game theory and have studied Schelling's work.

It is likely that the development of dynamic game theory will call for greater attention to both context and social psychology. In chess, the sequencing of moves is clear; in international bargaining over hostages or borders, moves are difficult to define and their sequence may be highly dependent on the course of play. Brams gives many interesting examples, including Samson and Delilah, Moses and Pharaoh, and Holmes and Moriarty, to bolster his account of the treatment of anticipation.

His concluding summary contains a nice synopsis of the new insights he claims to have provided in the treatment of stability, power and information. A better title for the book would have been *Modelling Moves and Anticipations in Some Dynamic Games*. □

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## New Journals Issue

This year, *Nature's* annual New Journals review supplement will appear in the issue of 29 September. Publishers and learned societies are invited to submit journals for review, taking note of the following criteria:

■ Journals that first appeared during or after June 1992 and issued at least four separate numbers by the end of April 1994 will be considered.

■ Journals covering any aspect of science are eligible, although those dealing with clinical medicine, engineering and pure mathematics are excluded, as are publications of abstracts.

■ Frequency of publication must be at least three times a year. The main language used must be English. Translation journals in English are, of course, eligible.

■ Deadline for submission is the end of May.

When submitting journals for review, please send at least four different issues (the first, the most recent and any two others) of each title, together with full details of subscription rates (personal and institutional) and frequency of publication to: Peter Tallack, *Nature*, 4 Little Essex Street, London WC2R 3LF, UK. For further information please telephone Peter Tallack on +4471-836-6633 (international), extension 2414.

# Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites

Eberhard H. Buhl<sup>\*</sup>, Katalin Halasy<sup>\*†</sup> & Peter Somogyi<sup>\*</sup>

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**Dual intracellular recordings from microscopically identified neurons in the hippocampus reveal that the synaptic terminals of three morphologically distinct types of interneuron act through GABA<sub>A</sub> receptors. Each type of interneuron forms up to 12 synaptic contacts with a postsynaptic principal neuron, but each interneuron innervates a different domain of the surface of the postsynaptic neuron. Different kinetics of the postsynaptic effects, together with the strategic placement of synapses, indicate that these GABAergic interneurons serve distinct functions in the cortical network.**

THE excitatory neuronal network in the hippocampus is controlled by inhibitory innervation that governs the threshold of activation<sup>1-4</sup>, pattern of action potential firing<sup>5-7</sup>, and modification of synaptic strength<sup>8</sup>. Such inhibition is largely mediated by fast-spiking, local-circuit cells that release GABA ( $\gamma$ -aminobutyric acid)<sup>4,7,9-12</sup>. In anatomical terms there are many different kinds of GABAergic neuron<sup>13-15</sup>, following several governing principles in their organization. First, the dendritic architecture of local-circuit cells reflects the spatial availability of afferent input<sup>16</sup>. Second, the axons of GABAergic cells vary with respect to targeting different domains on the surface of their respective postsynaptic target cells<sup>14,16-18</sup>. Third, the axonal terminal field of local-circuit cells may be precisely co-aligned with afferent excitatory inputs<sup>16,18</sup>. Although the latter features would suggest that it is the output, or, in physiological terms, the postsynaptic effect that is a key to the functional significance of interneurons, little is known as to which particular types of neuron mediate different inhibitory effects, such as recurrent inhibition<sup>1,2,6</sup>, population synchronization, or shunting of dendritic excitatory inputs<sup>19</sup>.

By combining electron microscopic analysis of synaptic connections with paired intracellular recordings from each of three distinct types of GABAergic cells and their respective postsynaptic principal neurons, we have found that each class of inhibitory neuron (axo-axonic, basket and bistratified cells) target different regions on the surface of principal neurons. Each type of inhibitory neuron forms between 6–12 synapses with an individual principal cell and each elicits short-latency inhibitory postsynaptic potentials (i.p.s.ps), but with different dynamic characteristics. GABA release from basket and axo-axonic terminals appears to be modulated by presynaptic GABA<sub>B</sub> receptors, whereas their postsynaptic effects are mediated by GABA<sub>A</sub> receptors.

## Diversity of interneurons and synaptic output

Intracellular recording and subsequent visualization of fast-spiking neurons with somata located in the cellular layers of the hippocampal formation revealed distinct stereotyped patterns of axonal and dendritic arborizations. Basket cells ( $n=31$  CA1;  $n=3$  CA3;  $n=5$  dentate gyrus (DG)) had most of their axon

TABLE 1 Location of synaptic release sites made by inhibitory cells on simultaneously recorded postsynaptic principal cells

| Presynaptic inhibitory neuron      | Type            | Location of release sites on a synaptically coupled postsynaptic cell |          |      |       | Overall random synaptic target pattern |                    |         |            |       |
|------------------------------------|-----------------|-----------------------------------------------------------------------|----------|------|-------|----------------------------------------|--------------------|---------|------------|-------|
|                                    |                 | Soma                                                                  | Dendrite | Axon | Total | Soma                                   | Dendrite           | Spine   | Axon       | Total |
| 1. Basket cell, CA1 (Fig. 1)       | Pyramidal cell  | 5                                                                     | 5        | 0    | 10    | 19 (44%)                               | 24 (56%)           | 0       | 0          | 43    |
| 2. Basket cell, CA1,               | Pyramidal cell  | 5                                                                     | 7        | 0    | 12    | 15 (45%)                               | 18 (55%)           | 0       | 0          | 33    |
| 3. Bistratified cell, CA1 (Fig. 2) | Pyramidal cell  | 0                                                                     | 6*       | 0    | 6*    | 4 (3+8%)*†                             | 27 (78%)           | 4 (11%) | 0          | 35    |
| 4. Axo-axonic cell, DG (Fig. 3)    | Granule cell    | 0                                                                     | 0        | 8    | 8     | 0                                      | 0                  | 0       | 33 (100%)  | 33    |
| Pooled data (incl. 1–4)            |                 |                                                                       |          |      |       | Soma                                   | Dendrite and spine |         | Axon       | Total |
| Basket cells ( $n=8$ )             | Pyramidal cells |                                                                       |          |      |       | 79 (50.6%)                             | 73 (46.8%)         |         | 4 (2.6%)   | 156   |
| Bistratified cells ( $n=2$ )       | Pyramidal cells |                                                                       |          |      |       | 4 (2+6%)*†                             | 45 (92%)           |         | 0 (0%)     | 49    |
| Axo-axonic cells ( $n=4$ )         | 3 p.c., 1 g.c.† |                                                                       |          |      |       | 6 (7%)                                 | 2 (2.5%)           |         | 76 (90.5%) | 84    |

\* Light microscopic estimate, three confirmed with EM.

† One on a pyramidal cell, three on a GABA-positive cell.

‡ p.c., pyramidal cell; g.c., granule cell.



terminals in the cellular layers (Fig. 1C). Electron microscopic analysis of a random sample of synapses ( $n=156$ ) showed that they make synaptic contacts predominantly on somata (50.6%) and proximal dendrites (46.8%) of pyramidal cells (Table 1). In contrast, a cell that we term the bistratified cell ( $n=11$ , CA1) had relatively few terminals in the pyramidal cell layer of the hippocampus and extensively ramified in stratum radiatum and oriens (Fig. 2B), some axonal arbors covering the whole depth of stratum oriens and radiatum. Electron microscopic analysis of the efferent connections showed that of those synapses established on pyramidal cells, only 2% were on the soma and 98% on their dendrites (Table 1). Axo-axonic cells ( $n=11$ , CA1;  $n=2$ , CA3;  $n=5$ , DG) formed synapses exclusively or predominantly (90.5%;  $n=84$  synapses examined) on the axon initial segment of principal cells (Figs 3C, 4d). Basket and axo-axonic cells have been shown to contain GABAergic markers<sup>20,21</sup> and in the absence of physiological verification, were thought to be inhibitory. As bistratified cells constitute a novel cell type we tested the GABA content of nine terminals of an intracellularly recorded cell by immunocytochemistry. All terminals were strongly GABA-positive (Fig. 4f). Finally, apart from the majority of selective cell types described above, a small proportion of cells ( $n=6$ ) with transitional anatomical properties were also found in the CA1 area.

### Sources of GABA<sub>A</sub>-receptor-mediated i.p.s.ps

The synaptic effect of the three types of presynaptic GABAergic cells ( $n=14$ ) was determined in paired recordings with postsynaptic pyramidal or granule cells ( $n=28$ ), followed by the anatomical definition of the cell types and in several instances the synaptic connections (Table 1). All three cell types evoked short-latency i.p.s.ps, with rise and decay kinetics broadly similar to GABA<sub>A</sub>-receptor mediated synaptic potentials (Figs 1A, B, 2A and 3A). At membrane potentials between  $-65$  and  $-50$  mV, i.p.s.ps were hyperpolarizing with reversal potentials (either directly determined or extrapolated) ranging between  $-65$  and  $-78$  mV, indicating that chloride is probably the major charge carrier<sup>22</sup>. The i.p.s.ps evoked by axo-axonic and basket cells (Figs 1Ab, Bb and c, and 3Ab) had short latencies ( $<2$  ms from the peak of the presynaptic action potential) and fast rise times, demonstrating that, with respect to receptor type, the axon initial segment is similar to the somatic membrane, where GABA<sub>A</sub>-receptor-mediated responses dominate<sup>23</sup>. Indeed, pharmacological experiments with GABA-receptor blockers (Fig. 5) demonstrate that the release of GABA from presynaptic terminals of axo-axonic cells and basket cells activates GABA<sub>A</sub>-receptors in the postsynaptic membrane (Fig. 5Ad and Bd), whereas presynaptic GABA<sub>B</sub> receptors regulate transmitter release at these terminals (Fig. 5Ac and Bc). It appears that both axo-axonic and basket cells can be the joint source of the prominent i.p.s.ps recorded in the cellular layers of the hippocampus and postulated to be produced by synapses at or near the somata of principal cells<sup>2,5</sup>. Moreover, both i.p.s.ps are compatible with the time course of recurrent inhibition. Indeed, the electrophysiological (Fig. 1Ad and E) and anatomical (Figs 1E and 4c) demonstration of reciprocal synaptic connections between one of the basket cells and a pyramidal cell verifies the hypothesis<sup>2,6</sup> that the basket cell is involved in recurrent inhibition.

### A source for pathway-specific inhibition

In contrast to the other cell types, the i.p.s.ps elicited by bistratified cells ( $n=4$  postsynaptic pyramidal cells) were of dendritic origin with considerably longer rise and decay parameters. Basket cell i.p.s.p. ( $n=11$  postsynaptic pyramidal cells) rise-times (10–90%) were, on average,  $4.7 \pm 2.2$  ( $\pm$ s.d.) ms versus  $13.8 \pm 5.6$  ms for those evoked by bistratified cells, and i.p.s.p. durations at half-amplitude were  $34.2 \pm 21.9$  ms versus  $67.1 \pm 20.7$  ms, respectively. Both parameters were significantly different ( $P < 0.05$ ; Mann–Whitney *U*-test). Although the slower kinetics of the i.p.s.ps evoked by the bistratified cell may reflect

the more distal placement of the synaptic release sites (considered here as the functional equivalent of synaptic junctions; the term 'bouton' generally denotes a vesicle-filled axon terminal of presynaptic cells) and therefore some degree of electrotonic attenuation (Fig. 2C), recent data<sup>24</sup> would additionally suggest the presence of a different subset of GABA<sub>A</sub> receptors in the dendritic region. A separate source of GABA released from bistratified cells at proximal dendritic receptors may thus provide a mechanism for pathway-specific control of excitatory inputs<sup>16,17,24</sup>, for example through dendritic shunting<sup>19</sup>. This hypothesis is supported by the co-alignment of the GABAergic bistratified cell axon with the glutamatergic Schaffer collateral input to the dendrites of pyramidal cells. As bistratified cells are strongly activated by Schaffer collaterals (data not shown), the release of GABA to dendrites will spatially and, to some extent, temporally coincide with the release of glutamate from Schaffer collaterals. Therefore it may be predominantly dendritic inhibition that will lead to an increase in the dynamic range of excitatory inputs<sup>17,25</sup>.

### Basket-cell-evoked post-inhibitory facilitation

Some degree of variability of averaged postsynaptic responses has been noted which was also attributed to different types of presynaptic cells<sup>10</sup>. The difference seen here in responses to basket and bistratified cells provides evidence for this assumption. However, we also found variability in the averaged responses of different cells postsynaptic to the same presynaptic inhibitory cell. For example, i.p.s.ps elicited by the same presynaptic basket cell in different pyramidal cells could either slowly decay back to baseline or display post-inhibitory facilitation (3 out of 15), which could be of sufficient amplitude to trigger action potentials (Fig. 1Bb, c and f). Depolarizing potentials have been reported in response to GABA application to the dendritic region of pyramidal cells<sup>26</sup>, and they are thought to be due to a more positive chloride equilibrium potential at the dendrites compared to the soma<sup>27</sup>. In light of the proximal placement of basket cell synapses, as well as the more distal position of the bistratified synapses, other mechanisms may underlie the depolarizing responses evoked by basket cells. Regardless of the ionic mechanism responsible for evoking post-inhibitory facilitation, the estimated (see below) convergence of 25 basket cells acting together on a single pyramidal cell may suffice to trigger rebound firing.

### Several release sites mediate unitary i.p.s.ps

Evidence for the monosynaptic nature of the connections was obtained by electron microscopy, which also revealed the precise number of synaptic release sites responsible for postsynaptic responses (Fig. 4 and Table 1). Two basket cells made 10 and 12 synaptic junctions respectively and one axo-axonic cell made 8 junctions on a single postsynaptic cell. Moreover, single presynaptic boutons may establish multiple synaptic release sites, raising the question of whether transmitter release at these sites is independent. In the case of the bistratified cell, following light microscopic prediction of six contacts, three out of three tested contacts were verified by electron microscopy to confirm the correctness of such an estimate.

In addition to multiple innervation from individual inhibitory cells, the postsynaptic cells also received substantially more synapses from non-labelled boutons which were similar to the identified ones, indicating convergence of inhibitory cells. Continuous serial sectioning of the postsynaptic cells for electron microscopy showed that the pyramidal cell postsynaptic to basket cell no. 2 received 119 synaptic contacts on the soma, in addition to the five made by biocytin-filled terminals. Provided that all these synapses were made by similar basket cells, approximately 25 basket cells may converge on a single CA1 pyramidal cell. Likewise, the axon initial segment of the granule cell (Fig. 3B) received forty synapses in addition to the eight made by biocytin-filled terminals (Figs 3C and 4d). This indi-

cates that about six axo-axonic cells converge on one granule cell.

## Discussion

Dual recording and labelling experiments revealed that three distinct types of hippocampal GABAergic interneurons elicit short-latency i.p.s.ps in postsynaptic principal cells. Recurrent collaterals of the latter evoke monosynaptic e.p.s.ps in pyramidal<sup>28</sup> and local-circuit cells<sup>28–30</sup>. In striking contrast to the recurrent pyramidal-to-interneuron connections, which are mediated by one synaptic junction<sup>30</sup> (Figs 1*E* and 4*c*), inhibitory

unitary interactions are generally mediated by multiple synaptic release sites, a finding that may also explain the safety of transmission, as evident from the rarity of apparent transmission failures<sup>7,10</sup>. Pharmacological data of unitary interactions suggest that at least basket and axo-axonic cells evoke their postsynaptic effects solely through GABA<sub>A</sub> receptors, thus suggesting synaptic segregation of GABA<sub>A</sub> and GABA<sub>B</sub> receptors on the surface of postsynaptic cells<sup>4,31–33</sup>.

The results reveal a division of labour among inhibitory interneurons which not only use the same transmitter, GABA, but also act through similar postsynaptic receptor mechanisms.

**FIG. 1** Postsynaptic effect of basket cells and location of synaptic release sites on a pyramidal cell in the CA1 area. In Figs 1–3, the soma, dendrites and intracellularly recorded membrane potential of presynaptic inhibitory cells is shown in red, their axon in yellow, and the synaptically coupled postsynaptic principal cell and its membrane potential in blue. **A, B**, Two identified basket cells (Table 1) elicit short-latency i.p.s.ps in postsynaptic pyramidal cells (**Ab** and **c**; **Bb–d, f**). Individual i.p.s.ps showed considerable amplitude fluctuation (**Bd**) and varied between the level of detectability and 2 mV. The amplitude of averaged basket cell i.p.s.ps ranged between 310 and 920  $\mu$ V and was correlated with the membrane potential of the postsynaptic cell. In basket cell 1 (shown in **C** and **D**) the i.p.s.p amplitude declined linearly between –50 and –60 mV and response reversal was extrapolated to –65 mV. Interestingly, i.p.s.ps could either decay back to baseline (**Ab, Bb**) or, alternatively, showed a depolarizing overshoot (**Bc**) of sufficient amplitude to trigger action potentials (**Bf**). The postsynaptic pyramidal cell illustrated in **C** and **D** elicited a reciprocal short-latency e.p.s.p. (amplitude 660  $\mu$ V) in the presynaptic inhibitory basket cell 1 (**Ae**), demonstrating that basket cells participate in recurrent inhibitory circuits. The traces in **Aa, b, d** and **e** and **Ba–c** represent averages of >100 sweeps. **C**, Most of the axon of this basket cell (no. 1 in Table 1) is restricted to the pyramidal cell layer (Str. pyr.), resulting in a general target profile rich in axosomatic synaptic release sites, also demonstrated at the single cell level in **D**. **D**, Location of 10 synaptic junctions (see Fig. 4 for example) by electron microscopy, received by the postsynaptic pyramidal cell shown in **C**, giving the postsynaptic effect shown in **Ab**. Three boutons (numbers 1, 3 and 7) made 2 synaptic junctions each. **E**, Enlargement of the area labelled by small arrow in **C**, showing a recurrent collateral of the pyramidal cell axon (ax) which is in contact with a basket cell dendrite. Subsequent electron microscopy revealed that a single synaptic release site (arrow; also shown in Fig. 4*c*) mediated the recurrent e.p.s.p. shown in **Ae**. Str. lac. mol., stratum lacunosum moleculare; Str. rad., stratum radiatum; Str. or., stratum oriens. Scale bars in **A** and **B**: **Aa, d** and **Ba**: 40 mV; **Be** and **f**: 20 mV; **Ac** and **Bd**: 5 mV; **Ab, e** and **Bb, c**: 1 mV; **A** and **Ba–d**: 50 ms; **Be** and **f**: 100 ms.

**METHODS.** The slicing procedure has been described<sup>40</sup>. In brief, recordings were obtained in 400- $\mu$ m-thick transverse hippocampal slices of adult (>150 g) female Wistar rats. Slices were maintained at 34–35 °C on a nylon mesh at the interface between oxygenated ACSF (in mM: 126 NaCl, 3.0 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, 2.0 MgSO<sub>4</sub>,

2.0 CaCl<sub>2</sub>, and 10 glucose) and a humidified atmosphere saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The flow rate was adjusted to 1.5 ml min<sup>–1</sup>. Micropipettes were filled with 2% biocytin in 1.5 M KCH<sub>3</sub>SO<sub>4</sub> and bevelled to a d.c. resistance ranging between 60–150 M $\Omega$ . Presumed interneurons were impaled using previously established physiological criteria<sup>9,40</sup>. When a stable recording was obtained, a search was made for synaptically coupled cells displaying the properties of hippocampal principal cells. Capacitive coupling between the recording electrodes was either eliminated off-line<sup>10</sup> or, alternatively, on-line using a modified Axoprobe amplifier. Synaptic coupling was tested using on-line averaging and injecting depolarizing current pulses into the presumed presynaptic cell, or injecting a constant depolarizing current which was adjusted to induce tonic firing at rates varying between >100 and 0.5 Hz. Diffusion of biocytin during most recordings usually resulted in adequate filling. Slices were processed for light and electron microscopy as before<sup>16,40</sup>. Recovered cells were reconstructed from serial 60- $\mu$ m sections using a light microscope and drawing tube at 1,250 $\times$  magnification.

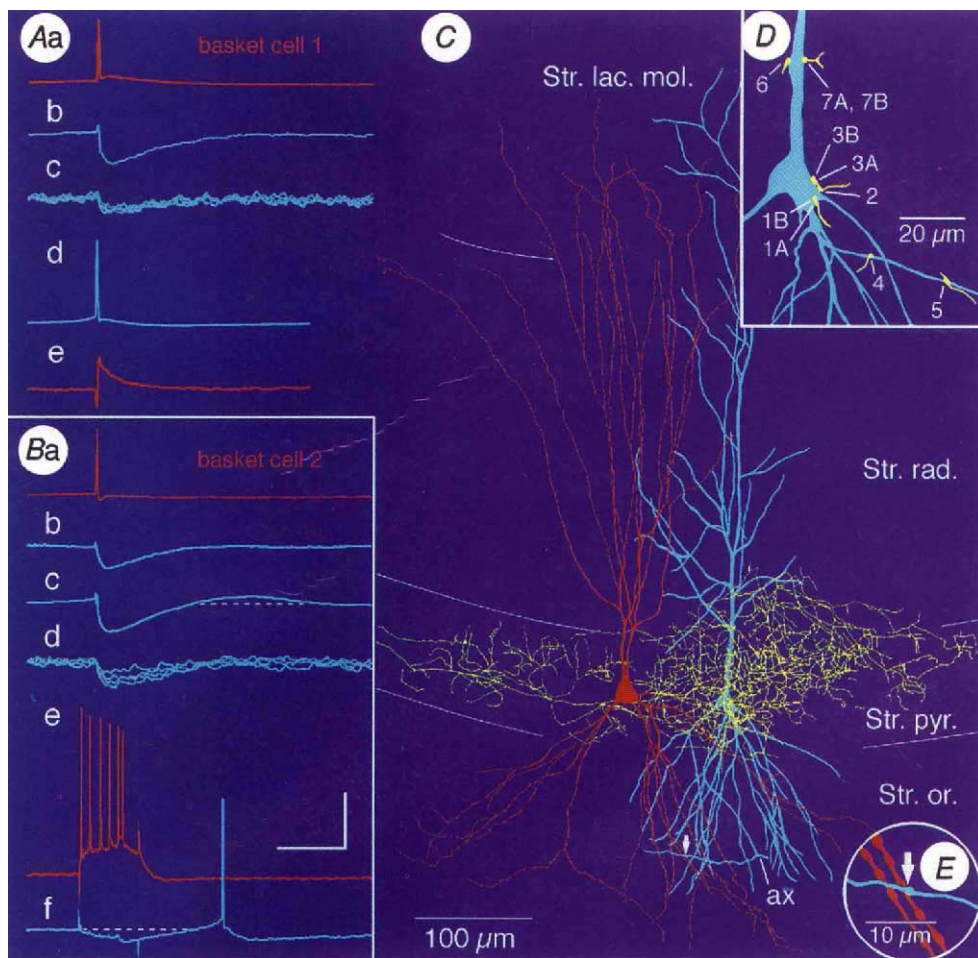




FIG. 2 Postsynaptic effect of a bistratified cell and location of contact sites on a pyramidal cell in the CA1 area. **A**, Action potentials (Aa) of this cell (Table 1, no. 3) elicited a small-amplitude, short-latency i.p.s.p. in a postsynaptic pyramidal cell (b). With linear regression, the i.p.s.p. reversal was extrapolated to  $-77$  mV. **B**, Same bistratified/pyramidal pair as in **A**; note the relatively sparse inhibitory axon (yellow) in stratum pyramidale (Str. pyr.) and its increased density in the adjacent strata radiatum (Str. rad.) and oriens (Str. or.), where Schaffer collateral afferents from the CA3 area terminate. Dendrites of this cell type do not significantly enter into the stratum lacunosum moleculare (Str. lac. mol.) where the perforant path terminates. **C**, Location of 6 contact sites between the GABAergic axon (yellow; see Fig. 4) and the postsynaptic pyramidal cell. Only the dendrites were contacted, and axo-somatic synapses were also rare in the random sample of synapses (Table 1).

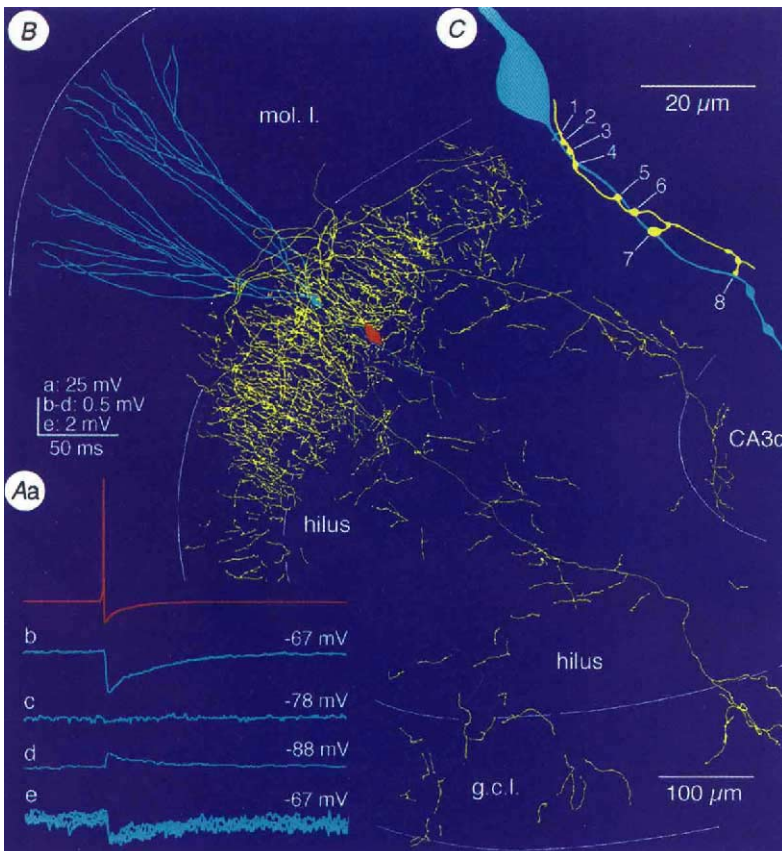
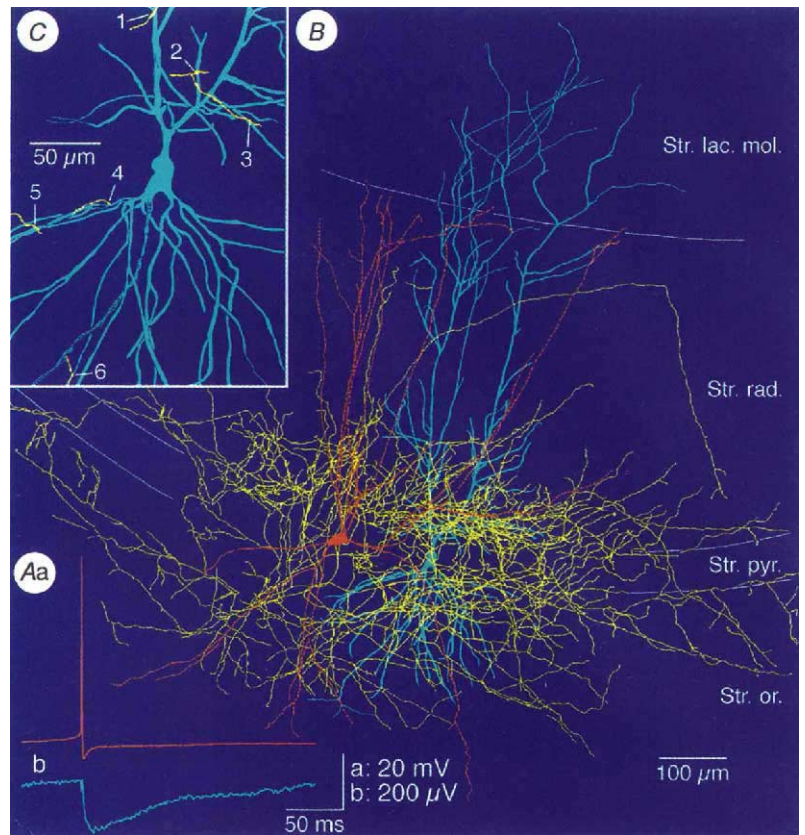


FIG. 3 Postsynaptic effect of an axo-axonic cell and location of 8 synaptic release sites on a granule cell axon initial segment in the dentate gyrus. **A**, At  $-67$  mV, the presynaptic axo-axonic cell (a) elicited a short-latency fast i.p.s.p. (b). Individual i.p.s.ps fluctuated in amplitude, which could be up to 2 mV (e), whereas the average of all trials had an amplitude of 460  $\mu$ V. The response reversed around  $-78$  mV (c and d), but the  $I/V$  relationship of the i.p.s.p. was clearly nonlinear. **B**, The inhibitory axon (yellow) innervated most of the granule cell layer (g.c.l.), the hilus, and the tip of the CA3c area. The dendrites of this axo-axonic cell were not recovered, only the cell body giving rise to the axon is shown. The postsynaptic granule cell received synapses only on the axon initial segment and a random sample of synapses confirmed this high degree of target specificity (no. 4 in Table 1). **C**, Location of 8 synaptic junctions (Fig. 4d) that mediated the effect shown in **A**. mol., Molecular layer.

FIG. 4 *a* and *b*, Electron microscopic demonstration of physiologically identified inhibitory synapses (arrows) between presynaptic boutons of the basket cell (Bt) and the soma (Pc; cell shown in Fig. 1) or the apical dendrite (Pd; no. 2 in Table 1) of pyramidal cells; *c*, Recurrent excitatory synapse between the pyramidal cell terminal (Pt; cell shown in Fig. 1) and a basket cell dendrite (Bd). Note the asymmetric postsynaptic density. Asterisk denotes an unlabelled terminal which is in asymmetrical synaptic contact (double arrow) with the same basket cell dendrite. *d*, Inhibitory synaptic contact between a bouton of the axo-axonic cell (Aat; no. 5 in Fig. 3C) and the axon initial segment (Gcais) of the granule cell shown in Fig. 3; asterisk marks a converging terminal from another axo-axonic cell; *e* and *f*, Synaptic junction between a bouton (Bit) of the bistratified cell shown in Fig. 2, and a non-recorded pyramidal cell dendrite (Pd). The section in *f* is serial to that in *e* and shows that the bistratified cell bouton is immunopositive for GABA, as demonstrated by the high density of metal particles. Scale bar for all pictures, 0.2  $\mu$ m.

**METHODS.** Resin-embedded cells were serially sectioned for electron microscopy and stained with lead citrate. Some sections were immunoreacted for GABA using a highly sensitive silver-intensified immunogold method<sup>17</sup> and a rabbit antiserum to GABA<sup>41</sup>. Synaptic contacts were identified on the basis of the rigid apposition of pre- and postsynaptic membranes, the electron-dense cleft material and the postsynaptic density, when detectable.

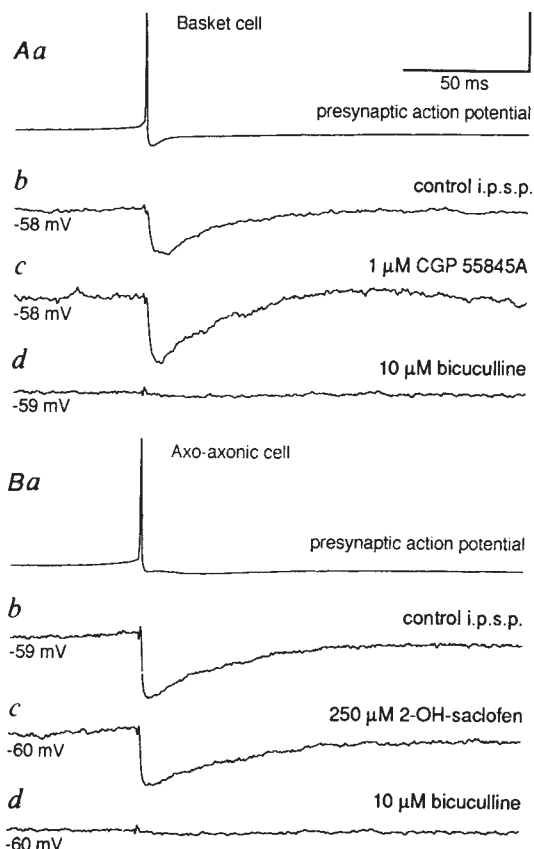
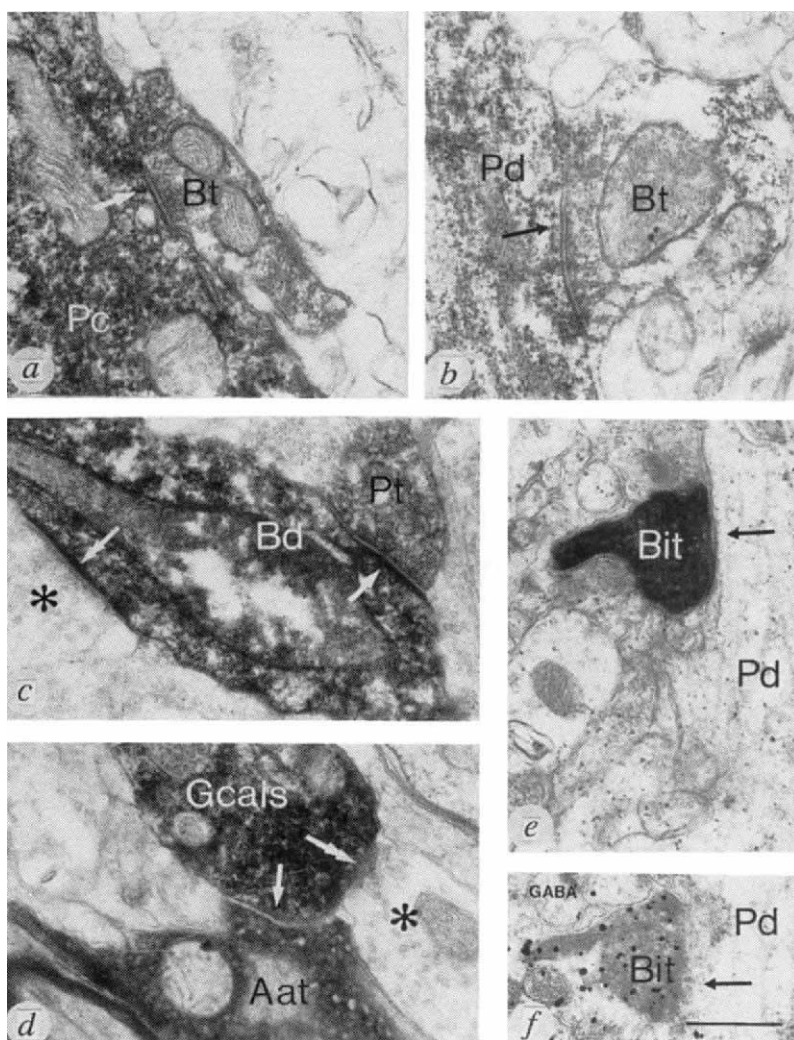


FIG. 5 Effect of GABA receptor antagonists on unitary i.p.s.ps in the CA1 region. **A**, A presynaptic CA1 basket cell (*a*) elicited a short-latency i.p.s.p. (*b*) in a postsynaptic pyramidal cell. Subsequent bath application of the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5  $\mu$ M) and the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (AP5; 30  $\mu$ M) left the response unaffected (not illustrated). Following superfusion of 1  $\mu$ M of the GABA<sub>B</sub> receptor antagonist CGP 55845A (ref. 42), the i.p.s.p.-amplitude increased by 33% (*c*), presumably as a result of an antagonist effect of CGP 55845A on presynaptic GABA<sub>B</sub> receptors<sup>43</sup>. Addition of 10  $\mu$ M bicuculline methochloride to the superfusate virtually eliminated the response (*d*). Input resistance (42 M $\Omega$ ) and time constant (11.4 ms) of the pyramidal cell were monitored throughout and remained stable. Scale bar (vertical): *a*, 25 mV; *b*–*d*, 0.5 mV. **B**, A CA1 axo-axonic cell (*a*) elicited a short-latency i.p.s.p. in a postsynaptic pyramidal cell (*b*). In the presence of 5  $\mu$ M CNQX and 30  $\mu$ M AP5, bath application of the GABA<sub>B</sub> receptor antagonist 2-OH-saclofen (250  $\mu$ M) resulted in a 12% decrease of the i.p.s.p. amplitude (*c*). In view of the known slow onset of GABA<sub>B</sub>-receptor-mediated postsynaptic mechanisms<sup>44</sup>, this effect on the early phase of the i.p.s.p. is probably due to a partial agonist action of 2-OH-saclofen on presynaptic GABA<sub>B</sub> receptors<sup>45</sup>. A –1 mV change of the membrane potential during the recording may have also contributed to the amplitude decrease of the i.p.s.p. Subsequent perfusion with 10  $\mu$ M bicuculline methochloride eliminated the response (*d*). The input resistance of the pyramidal cell at the beginning and end of the experiment remained unchanged (24 M $\Omega$ ). All traces represent averages of >100 sweeps. Scale bar (vertical): *a*, 25 mV; *b*–*d*, 0.5 mV.



However, kinetic differences of the postsynaptic effects, as well as the anatomical segregation of release sites, indicate that GABA<sub>A</sub> receptor properties, as well as the placement of synaptic release sites, play a role in the functional differentiation of inhibitory neurons. In this respect, the strategic location of GABA<sub>A</sub> receptors on the axon initial segment, where the action potential threshold is lowest<sup>34</sup>, predestines axo-axonic cells to govern the firing threshold, as suggested earlier on anatomical and physiological grounds<sup>35,36</sup>. Basket cells not only mediate recurrent inhibition, they also evoke rebound firing and, by virtue of their divergence to hundreds of pyramidal cells, may be an effective means to synchronize principal cell activity, as predicted on theoretical grounds<sup>37</sup>. In the living animal, such GABA<sub>A</sub>-

receptor-mediated mechanisms are involved in rhythmic membrane potential changes, which may result in the phase-locking of large numbers of principal cells<sup>38</sup>. In contrast to axo-axonic and basket cells, the bistratified input discovered here is selectively associated with a major glutamatergic input, the Schaffer collateral/commissural pathway. This selective alignment of GABAergic synapses with particular excitatory afferents on the same dendritic segments was previously found in the dentate gyrus<sup>16,17</sup> and also in the CA3 region<sup>18</sup>. This spatial coincidence provides a selective mechanism for bistratified cells to carry out a downward re-scaling of Schaffer collateral/commissural e.p.s.ps in pyramidal cells. Differences in function may therefore be the key to explain the diversity of cortical interneurons<sup>39</sup>. □

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## LETTERS TO NATURE

### The structure of the Virgo cluster of galaxies from Rosat X-ray images

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THE Virgo cluster<sup>1–10</sup> is the nearest large concentration of galaxies to us, and therefore plays a critical role in calibrating the cosmological distance scale<sup>11</sup>. Unfortunately, the cluster is very irregular, making it difficult to model the distribution of mass within it, and therefore impossible to determine its content of dark matter. X-ray observations offer a way around this problem, by revealing the hot, diffuse gas that is thought to fill the cluster's gravitational potential well in a way that closely matches the total distribution of mass<sup>12</sup>. Here we report X-ray images of Virgo, obtained with the Rosat observatory, which show hot luminous gas extending over most of the optically visible cluster. The data show that a large part of the mass of the cluster is centred on the galaxy M87, with smaller concentrations around M86 and M49. Our results for M86 support the recent suggestion<sup>3</sup> that it is part of a small group of galaxies that is merging with the main cluster.

The X-ray emission from clusters is a good tracer of the dynamical structure and the mass distribution in the cluster: for example, irregular or multiply peaked X-ray emission in some clusters is a clear signature that these clusters are still evolving

towards a more symmetrical equilibrium configuration<sup>13,14</sup>. Previous X-ray observations of the Virgo cluster in the central region around M87 with the Einstein and Exosat observatories allowed a study of the halo region of M87 out to a radius of ~100 arcmin<sup>5,6,15</sup> (~0.6 Mpc, for a Virgo distance of 20 Mpc which is adopted throughout this paper). These results, and a few other Einstein observations towards Virgo, combined to a small mosaic showed that the hot gas extends even further out<sup>6</sup>. A first indication of diffuse emission extending over ~10° in the sky was found with the non-imaging collimator detector on board the Ginga satellite<sup>7</sup>.

The Virgo region was scanned during the Rosat all-sky survey<sup>16,17</sup> between November 1990 and January 1991 with an average exposure time of ~450 s. The position sensitive proportional counter (PSPC) with low energy resolution and a spatial resolution of about 20–60 arcsec (on-axis, detector average) was used as detector<sup>18</sup>. The PSPC in combination with the X-ray telescope offers an energy window from 0.1 to 2.4 keV. Figure 1a and b show contour maps of the X-ray surface brightness of a 12.8 × 12.8° area of the Virgo cluster region in a hard (0.4–2.4 keV) and soft (0.1–0.4 keV) energy band, respectively. To display the full dynamic range (up to 3,000) of these images, they have been filtered with a set of gaussian filters where the filter scale was varied depending on the brightness of the image pixels. For the faint emission a maximum smoothing scale with a full width at half maximum (FWHM) of 55 arcmin for the gaussian was used, whereas point sources are only marginally broadened.

Whereas the soft-energy-band image is dominated by foreground emission from gas at ~10<sup>6</sup> K in our Galaxy<sup>19</sup>, most of the cluster emission originating from gas at temperatures of sev-

eral tens of million K is received in the hard energy band. Therefore the hard-band data provide the highest signal-to-noise ratio for the cluster structure. The most prominent features in Fig. 1a are the well known giant X-ray halo around M87, and the haloes around N4406 (M86)<sup>6,20</sup> and N4472 (M49)<sup>6,21</sup>. Also, the Virgo elliptical galaxies N4261, N4374 (M84), N4552 (M89) and N4649 (M60), and the spiral galaxies N4325, N4579 (M58) and N4639 are readily identified as individual X-ray sources<sup>22</sup>. Most of the other X-ray sources are foreground stars, background quasars and three Abell clusters. Faint X-ray emission can be traced out to a distance of  $4-5^\circ$  ( $\sim 1.5-1.8$  Mpc) from M87. An exception is the western side, where the emission falls off more steeply. This sharp edge does not correspond to a strong gradient in the galactic hydrogen column density which could have caused this feature by absorption<sup>23</sup>. In the southern direction the X-ray emission is more extended, even beyond M49. The northern edge of the cluster is more irregular and the cluster boundary seems to dissolve into several individual clumps, which appear clearly more extended than some obvious point sources in the same area. In the southeastern quadrant of the image, the North Polar spur (the rim of a hot galactic superbubble) is contributing to the X-ray surface brightness and is contaminating the cluster image. This structure (that is related to plasma at  $\sim 10^6$  K) is more pronounced in the soft-band image, Fig. 1b. Thus most of the emission in the lower-left corner is not related to the Virgo cluster, as indicated in the figure.

The X-ray morphology is very similar to the structure in the galaxy distribution as (for example) observed in the photometric survey by Binggeli, Tammann and Sandage<sup>1,2</sup>, which can be seen for the first time in this large-scale X-ray image. A contour map of the Virgo galaxy density as derived in this survey is underlaid in grey scale in Fig. 1a. This close correspondence could be expected because both cluster components are tracing the same

mass distribution. In both wavelength regions, the major part of the cluster consists of the A cloud around M87 and the smaller B cloud around M49 (as designated by Binggeli *et al.*). The asymmetrical extension to the eastern side and the sharp western edge are also prominent in both cases. The fact that the M49 halo is so much smaller in the X-ray image compared to the optical appearance of the B system, is noted as a striking difference in the two surveys. In the optical survey the A and B clouds are surrounded by more loose clouds of galaxies on larger scale<sup>2,4,24</sup>, but at the sensitivity of the Rosat all-sky survey no significant emission is detected from these regions. These clouds are composed largely of spiral galaxies<sup>24</sup>, and predominance of spiral galaxies is commonly found in regions where the density of X-ray luminous gas is not high.

In an attempt to analyse the structure of the Virgo cluster in more detail, we decomposed the X-ray surface brightness distribution into several components involving spherically symmetric haloes around M87, M86 and M49. The surface brightness profiles of these haloes are fitted by three-parameter analytical models (modified isothermal self-gravitating gas models, see ref. 25) with results shown in Fig. 3a. A two-dimensional, spherically symmetric image was then subtracted from Fig. 1a and the resulting residual X-ray brightness image is given in Fig. 2. There are only two small regions with negative contours at the  $-3\sigma$  level (shown as dashed lines) in the southwest of M87 where the brightness gradient is steepest and in the centre of the M87 halo due to a small asymmetry. This illustrates the validity of the decomposition. The cluster A 1552 which was hidden in the X-ray halo of M87 before is now clearly seen in the residual image. Thus from the total X-ray luminosity of  $\sim 8.3 \times 10^{43}$  erg s<sup>-1</sup> in the 0.1–2.4 keV band, 71% originates from the M87 halo, 9% can be attributed to the haloes of M86 and M49, and up to 5% may come from point sources not related to the cluster. Most

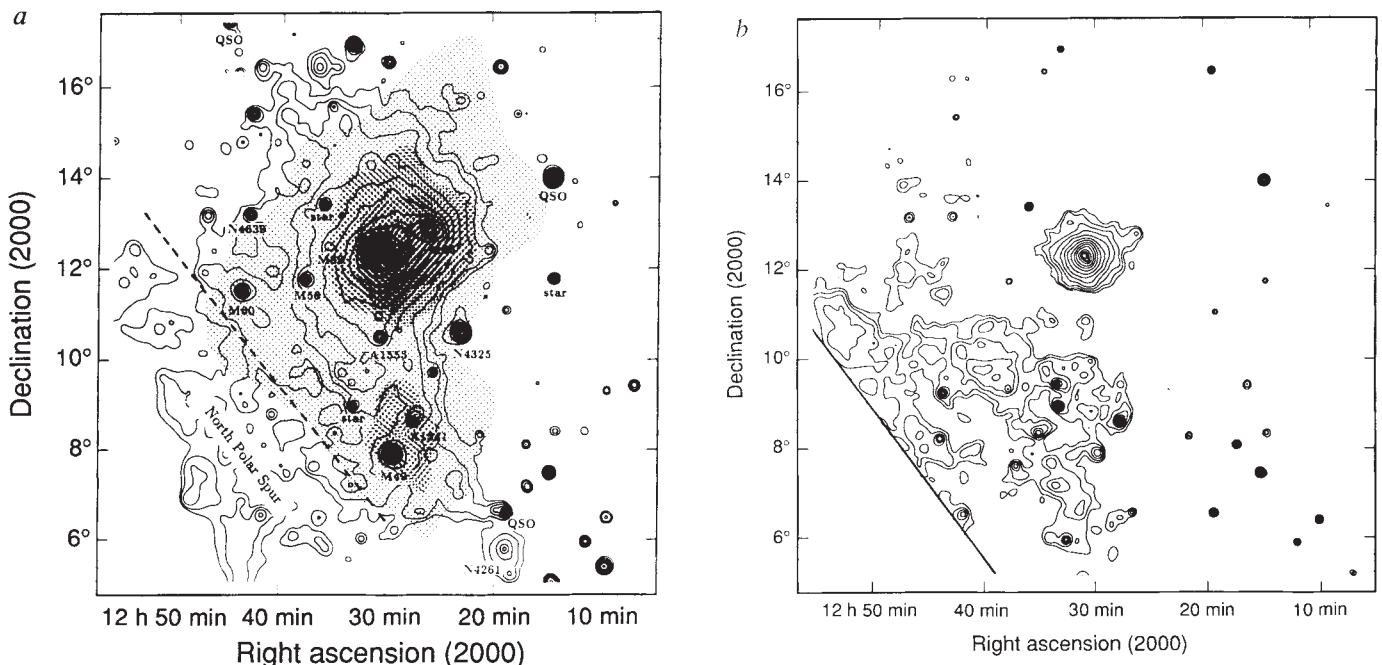


FIG. 1 a, Contour plot of the X-ray image of the Virgo cluster region in the Rosat all-sky survey in the hard energy band (0.4–2.4 keV). The first two contours correspond to the  $3\sigma$  and  $5\sigma$  levels above the background noise; the following contour levels increase by factors of 1.5. The image has been smoothed with a variable gaussian filter with a FWHM of 55 arcmin on the faintest levels and decreasing filter size with increasing surface brightness. Several Virgo cluster galaxies and some foreground and background sources are identified. The galaxy density distribution as taken from the photometric survey of Binggeli *et al.*<sup>2</sup> is shown in

grey scales. b, Contour plot for the same region of the photons received in the energy band 0.1–0.4 keV. The same filtering has been applied as in a. Less X-ray emission from the cluster is received in this energy band, but the core region of the M87 halo is still prominent. The feature in the lower-left corner is X-ray emission from the North Polar spur (a galactic superbubble feature). It is also contaminating the hard-band cluster image as indicated in a. No data were analysed here for the region in the lower-left corner.



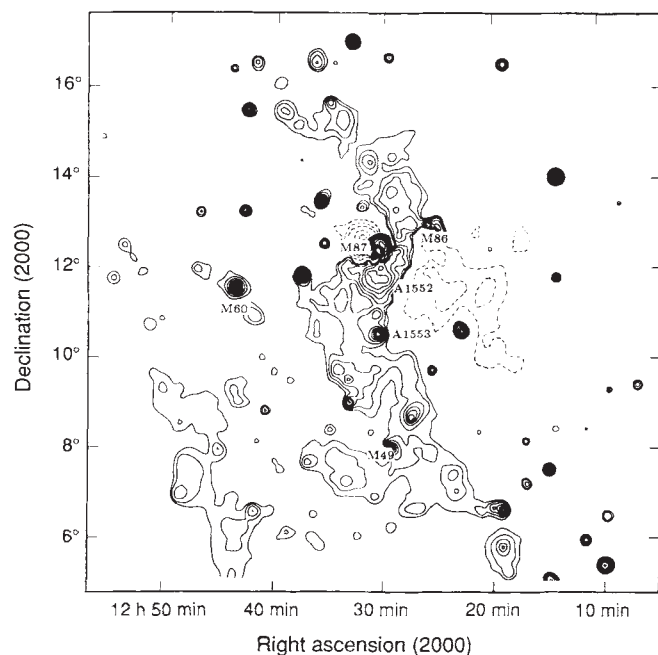


FIG. 2 Contour plot of the residual X-ray surface brightness image that is obtained by subtraction of a model image corresponding to the model fit of Fig. 3a from Fig. 1a. The contour levels are the same as in the previous images. Negative contours are shown as dashed lines. Only at the sharp edge on the western side is the density of the X-ray luminous gas significantly overestimated by the model.

of the remaining  $\sim 15\%$  of the flux is contributed by the asymmetrical, low-surface-brightness emission of the cluster.

Spectra were analysed for photons from concentric rings around M87 with outer radii of 5, 10, 20, 60, 120 and 180 arcmin. For the temperature determination the spectra were fitted by theoretical model spectra obtained with the Raymond-Smith code<sup>26</sup>, with three important fit parameters; gas temperature, metallicity and absorbing interstellar hydrogen column density.

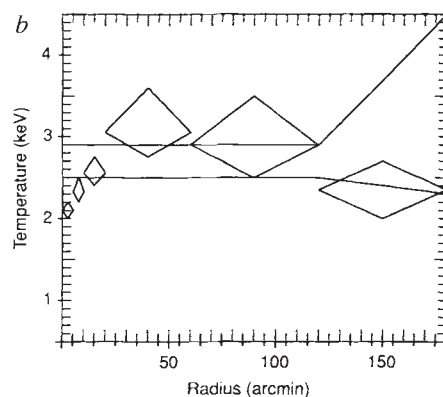
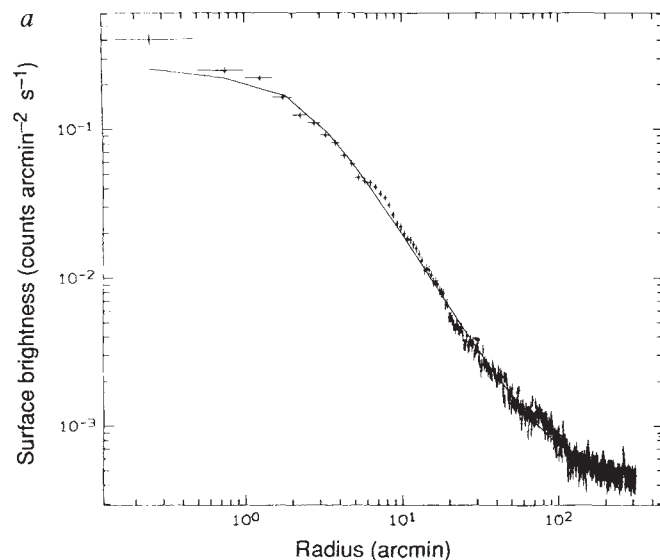


FIG. 3 a, Azimuthally averaged surface brightness profile for the X-ray halo around M87 (data points) and result of a fit of isothermal models<sup>25</sup> to the data (line). The shape of the model curve is determined by the fit parameters: core radius = 2.3 arcmin and slope = 0.86. In these data the core is unresolved. b, Radial temperature profile of the X-ray luminous gas in the halo around M87. The diamonds give the values determined from the Rosat data, and the continuous lines show the results of a north-south scan of the Virgo region with the Ginga satellite. The symbols indicate  $1\sigma$  errors in the temperature determination.

For the two innermost rings which cover the brightest area, good temperature constraints and metallicities of 0.4–0.5 of the solar value were obtained. Due to the limited energy resolution of the Rosat PSPC and the small change of the spectral shape with temperature in the relevant energy range, better constraints for the temperature can be found by fixing some of the fit parameters. Therefore in the regions of fainter emission, we fixed the column density to the observed Galactic value of  $\sim 2.5 \times 10^{20} \text{ cm}^{-2}$  and the metallicities to 0.44 for ring 3, 0.3 for ring 4 and 0.2–0.3 for rings 5 and 6 (according to the results of the Ginga scans of M87 where a decrease of the iron abundance with distance from M87 was found<sup>15</sup>). The resulting temperature profile is shown in Fig. 3b. The overall temperature determined for the region within  $1^\circ$  radius of M87 was  $2.4 [+0.30 - 0.2] \text{ keV}$ , in good agreement with the results from Ginga<sup>27</sup> and Exosat<sup>28</sup>. The significant decrease of the temperature inside a radius of 20 arcmin is perfectly consistent both with the existence of a cooling flow in the intracluster gas and with the temperature profile that has been deduced in earlier cooling flow models based on Einstein imaging data<sup>15</sup>. An indication of a temperature decrease in the cooling flow region was also already found in the spectral analysis of the Einstein observation<sup>5</sup>. The outer part of the temperature profile is consistent with a constant temperature. (The increase of the temperature found in the Ginga scans at  $\sim 2-3^\circ$  south of M87 is not reproduced by our data. It may possibly be caused by the background clusters A1552 and A1553 in the region.)

From these data an approximate gravitational mass estimate can be performed for the haloes of M87, M86 and M49. For outer radii of 1.8, 0.28 and 0.24 Mpc, respectively, the total masses of these subsystems are  $\sim (1.5-6) \times 10^{14}$  solar masses ( $M_\odot$ ) for M87 and of the order of  $(1-3) \times 10^{13} M_\odot$  for the two smaller haloes. The mass estimate for the M87 halo is based on azimuthally integrated surface brightness profiles in different sectors in which the X-ray emission can be traced out to  $\sim 1.8 \text{ Mpc}$  (except for the southwestern side). The detection of the intracluster gas dominantly in the harder band implies gas temperatures above  $\sim 0.5 \text{ keV}$ . Fitting popular models for the mass distribution to these constraints<sup>29,30</sup> yields the above mass estimate. For the smaller haloes, for which we find bulk gas temperatures of  $\sim 1.2 \text{ keV}$  (M49) and  $1.0 \text{ keV}$  (M86), the mass estimates are based on isothermal models and polytropic models

with a large range of temperature gradients. Due to the high velocity interaction of the gas haloes of M86 and M87, the M86 mass estimate is more speculative. A detailed account of the mass determination will be given in a forthcoming paper (H.B., S. C. Schindler and U.G.B., manuscript in preparation). The ratios of the gas mass to the total mass are then 7–36%, 3–8% and 1–3%, respectively. The central parts of the halo regions have also been targets of deeper Rosat pointed observations from which more precise information is expected.

These results have interesting implications. Much of the mass of the Virgo cluster is contributed by a massive cluster core that is centred on M87. The fact that it can be roughly fitted by a spherically symmetric model may imply that the core is close to a relaxed configuration. The small haloes around M86 and M49 have quite similar masses, but they differ in detail. M49 is more compact and has a small gas mass fraction, which is more typical of a giant halo around an elliptical galaxy<sup>21,31</sup>. M49 is located, however, in a larger region of diffuse emission that is associated with the B cloud of galaxies as shown in Fig. 1a. M86 has a shallower X-ray surface brightness profile and a larger gas mass fraction and thus resembles more a group of galaxies<sup>32,33</sup>.

M86 has usually been taken as the text-book example of a galaxy that is stripped from its interstellar medium during infall to a galaxy cluster<sup>20</sup>. The galaxy has a blue-shifted spectrum with velocity  $v = -227 \text{ km s}^{-1}$  and is presumably falling into the cluster from behind<sup>3</sup>. An analysis of the velocity dispersion of Virgo galaxies in the region around M86 by Binggeli *et al.*<sup>3</sup> has shown that there is an asymmetric blue-shifted tail in the distribution, consisting mainly of dwarf ellipticals, implying that M86 is actually part of a small group of galaxies. This implication is strongly supported by the present results. Thus the process we are witnessing here is most probably not the infall and stripping of a single galaxy but rather the merger of a small galaxy group with the Virgo cluster. □

## Synthesis of a fully unsaturated all-carbon ladder polymer

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For some time we have been trying to synthesize structurally perfect, fully unsaturated, double-stranded (ladder) polymers<sup>1</sup>; such polymers might combine favourable electronic properties with processability. Here we report the successful synthesis of the fully unsaturated ladder polymer **1a** (Fig. 1), by way of the Diels–Alder precursor polymer **7** (Fig. 4). The structure of **1a** closely resembles the hypothetical open-chain, polymeric analogue of the belt-region of the icosahedral  $C_{60}$  (refs 2, 3) molecule, **1b** (Fig. 1). Polymer **1a**, despite its extended  $\pi$ -conjugation, is stable in oxygen and may therefore be of interest for electroluminescence and photovoltaics applications<sup>4</sup>. Owing to both the relatively mild reaction conditions required for its generation and its double-stranded structure, we expect **1a** to have less interruptions of the  $\pi$ -molecular orbital delocalization along the backbone as compared with the single-stranded polymer poly(phenylene vinylene), which is presently the material of prime interest for the active elements in light-emitting diodes<sup>5,6</sup>.

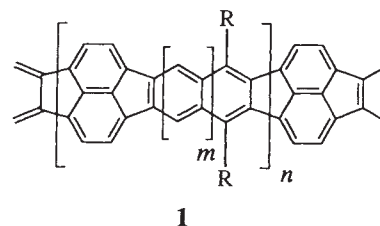
Each five-membered ring in the target polymer **1a** (Fig. 1) is joined to three six-membered rings as shown. The polymer should, therefore, have a planar, board-like structure. There is no doubt that the well known and often applied method to increase the solubility of conformationally rigid polymers by attaching flexible chains<sup>7</sup> will not be powerful enough to keep **1a** in solution, especially if high-molecular-weight material is concerned. The use of precursors has proved very successful in cases where the synthesis of insoluble and still well-defined polymers has to be brought about. Prominent examples are the Durham polyacetylene<sup>8</sup> and poly(phenylene vinylene) (PPV)<sup>9,10</sup>, where the first step is to make characterizable and processable polymers which are then, in a second step, converted cleanly to the final target polymers by some polymer analogous reaction. Very high conversions are necessary, but not sufficient.

In the initial phase of our project to synthesize polymer **1a**, the structurally related model compounds **4** and **5** were synthesized and studied with specific focus on the conversion of the transformation of **4** to **5** (Fig. 2). Most importantly, compound **4** upon reaction with the dehydrogenation agent dichloro(dicy-

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a:  $m = 0$ ;  $R, R = -(CH_2)_{12}-$   
b:  $m = 1$ ;  $R = H$

FIG. 1 The structures of the fully unsaturated target polymer **1a** and a hypothetical, linearly extended fragment of  $C_{60}$ , **1b**.



ano)benzoquinone (DDQ) gave **5** with a conversion of virtually 100% (isolated yield, 96%). The above-mentioned requirement for a reaction to be potentially useful for a polymer analogous application was thus fulfilled. Figure 3 shows the crystal structures of both model compounds; the relevant crystallographic data are given in Table 1. As expected, compound **4** has a kink caused by the sites of saturation, whereas compound **5** is nearly planar. From this model study it was concluded that polymer **7** (Fig. 4) would be a potential precursor for polymer **1a**.

The synthesis of polymer **7** could actually be achieved (Fig. 4). It is based on the polyaddition of the Diels–Alder AB-type monomer **6a** which proceeds with the elimination of one equivalent of carbon monoxide per addition step. The structurally related **6b** has already been described in the literature<sup>11</sup>. Attempts to polymerize it, however, met with little success. Besides some dimer and trimer, only insoluble and ill-defined material was obtained. Our route is superior for two reasons. First, monomer **6a** contains a flexible alkyl loop (*ansa* compound). This leads to a drastic increase in the solubility of the corresponding polymer and its growth is not prevented at a very early stage by insolubility. Second, there is the use of the antioxidant *o,o'*-di-*t*-butyl-*p*-methylphenol (**8**), the presence of which is essential to avoid side reactions. Without such a reagent the polymer is already partially dehydrogenated during growth, presumably by the monomer and cyclopentadienoid end-groups, a process which inevitably leads to termination.

Polymer **7** dissolved in chloroform gives yellow-orange solutions, whose ultraviolet spectra are very similar to those of model compound **4**. The structure proof rests basically upon its NMR spectra. Most informative is the <sup>13</sup>C NMR spectrum showing signals at  $\delta = 20\text{--}35$ , 51 and 120–145 p.p.m., which are assigned to the alkyl loop, the two saturated backbone carbons and the unsaturated backbone carbons, respectively. The signals in the <sup>1</sup>H NMR spectrum are broad and unstructured as is typical for Diels–Alder ladder polymers. Information about the relative orientation of the alkyl loops cannot be extracted at present. Size-exclusion chromatography investigations of solutions of **7** in tetrahydrofuran from independent preparations indicate monomodal molecular-weight distributions with  $2,400 < M_n < 5,100$  ( $7 < P_n < 14$ ),  $6,600 < M_w < 12,700$  ( $18 < P_w < 34$ ) and  $2.5 < D < 2.8$  (versus polystyrene standard, 20 °C). Here  $M_n$  is the number average of the molecular weight;  $M_w$  is the weight average of the molecular weight;  $P_n$  is the degree of polymerization corresponding to  $M_n$ ;  $P_w$  is the degree of polymerization corresponding to  $M_w$ ;  $D = M_w/M_n$  is the polydispersity index.

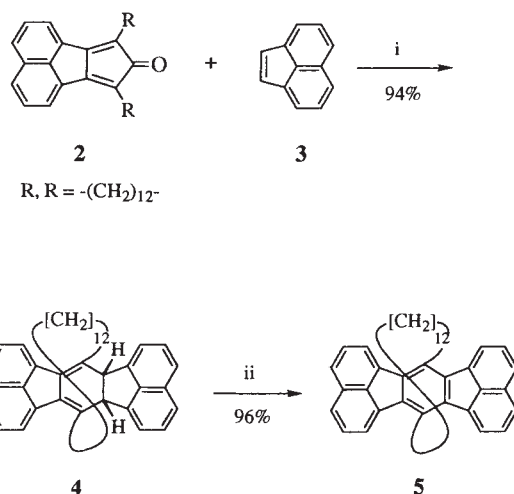


FIG. 2 Synthesis of model compounds **4** and **5** (reaction (i)) **2** (370 mg; 1 mmol, **3** (155 mg; 1 mmol), 110 °C, toluene, 24 h,  $-\text{CO}$ ; reaction (ii)) **4** (200 mg; 0.4 mmol), DDQ (140 mg; 0.6 mmol),  $\text{CH}_2\text{Cl}_2$ , 1 h, 20 °C).

The polymer analogous conversion of **7** into the target **1a** was carried out similarly to the one from **4** to **5**. Thus, in a typical dehydrogenation experiment, polymer **7** (200 mg, 0.55 mmol) dissolved in methylene chloride was reacted with DDQ (125 mg, 0.55 mmol) at room temperature. Immediately on addition of DDQ the colour of the solution changed from yellow-orange to deep red. After 4–6 hours a precipitate had formed which, together with some soluble oligomeric material, was recovered by precipitating the whole mixture into methanol, followed by filtration. The deep-red, amorphous solid obtained was washed with methanol to remove the hydroquinone (95% mass recovery). The structure of the material was established as **1a** on the basis of: (1) the correct data from elemental analysis (calculated: C, 92.3; H, 7.7, found: C, 92.2; H 7.9); (2) the transmission ultraviolet-spectrum (of low-molecular-weight material) in chloroform (Fig. 5), and (3) the solid-state <sup>13</sup>C NMR spectrum (Fig. 5). The ultraviolet spectrum of **1a** shows a considerable bathochromic shift of the maximum wavelength at which absorption

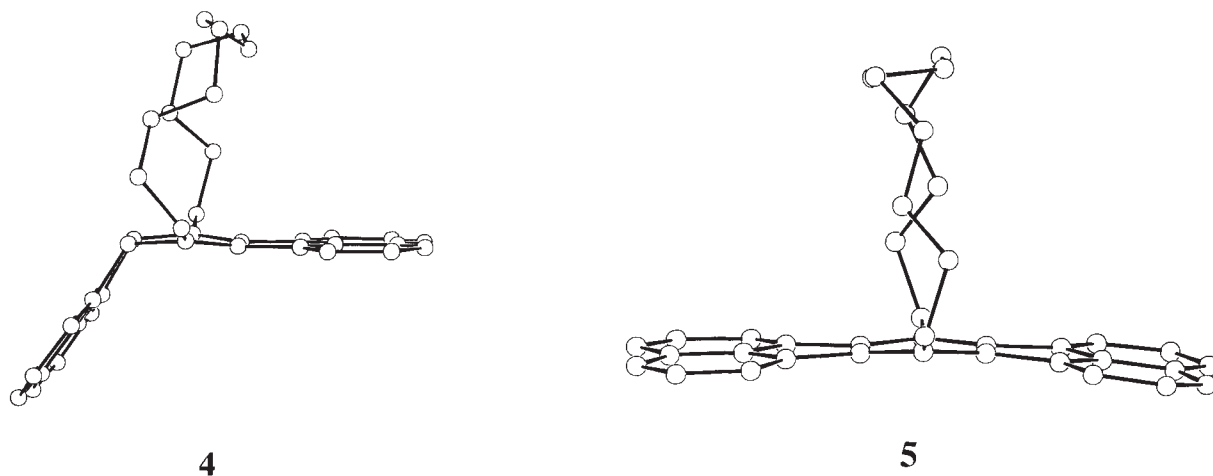


FIG. 3 Crystal structures (ORTEP) of model compounds **4** and **5**. Compound **4** has two areas of  $\pi$ -conjugation which are disrupted by the two

hydrogen-carrying saturated carbon atoms. These sites of saturation cause the structure to be kinked. In contrast, compound **5** is planar.

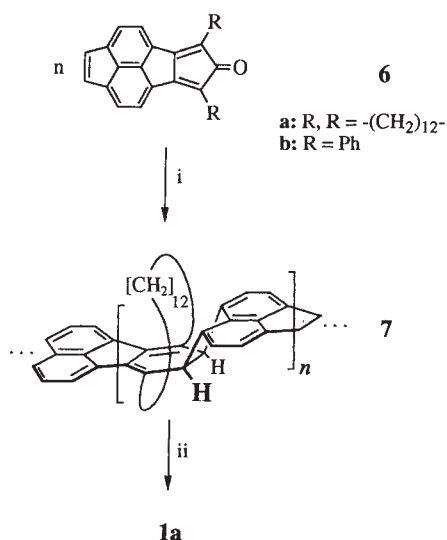


FIG. 4 Synthesis of precursor polymer **7**, and its conversion into the unsaturated target polymer **1a** (reaction (i) **6** (500 mg; 1.26 mmol), **8** (50 mg; 20 mol%), 110 °C, toluene, 24 h, 95% yield; reaction (ii) see text.

occurs as compared with models **4** and **5**, which agrees well with the proposed increase in  $\pi$ -conjugation. The visual appearance of solutions of **1a** and  $C_{60}$  is very similar. For the ultraviolet spectrum of  $C_{60}$  see ref. 12. The match between the cross polarization magic-angle spinning (CPMAS) spectrum of **1a** and the solution spectra of models **4** and **5** is excellent (Fig. 5). Most importantly, the signal of **4** at  $\delta = 50$  p.p.m., indicating saturated carbons in the backbone, does not appear in the spectrum of **1a**. The material can be exposed to air for weeks without any

TABLE 1 X-ray structure analyses of compounds **4** and **5**

| Compound                             | <b>4</b>                             | <b>5</b>                              |
|--------------------------------------|--------------------------------------|---------------------------------------|
| Habit, space group                   | Monoclinic, $P2_1/c$                 | Monoclinic, $P2_1/c$                  |
| <i>a</i> , <i>b</i> , <i>c</i> (Å)   | 17.896 (6), 10.9862 (19), 14.140 (8) | 9.1481 (5), 20.5659 (11), 14.1275 (6) |
| $\beta$ (degrees)                    | 109.309 (20)                         | 93.314 (5)                            |
| <i>V</i> (Å <sup>3</sup> ), <i>Z</i> | 2,624, 4                             | 2,653.5, 4                            |
| $\rho$ (g cm <sup>-3</sup> )         | 1.25                                 | 1.25                                  |
| No. of reflections                   | 3,504                                | 3,450                                 |
| No. observed                         | 1,776                                | 3,083                                 |
| <i>R</i> , <i>R</i> <sub>w</sub>     | 0.056, 0.061                         | 0.040, 0.046                          |

Equipment used: Enraf-Nonius CAD-4 diffractometer with Cu  $K\alpha$  radiation ( $\lambda = 1.5405$  Å, graphite monochromator), sample at room temperature. The structures were solved by direct methods (MULTAN). Empirical absorption correction, anisotropic temperature factors for O and C, refinement of the H atoms in the 'riding mode' with fixed isotropic temperature factors. Details of the crystal structure can be obtained from the Fachinformationszentrum Karlsruhe, Gesellschaft für wissenschaftlich-technische Information mbH, D-76344 Eggenstein-Leopoldshafen, depository number CSD-57962.

detectable change. As pointed out, the higher-molecular-weight fractions of polymer **1a** are insoluble in common organic solvents (for example, tetrahydrofuran, chloroform and toluene). This holds true, provided that all kinks (sites of saturation) are removed. If less than equimolar amounts of DDQ are used, partially planarized material is obtained which is still soluble. In other words, there seems to be an optimum between uninterrupted  $\pi$ -conjugation on the one hand and solubility on the other. This issue is presently under investigation because it has implications for the ability of this material to form a film.

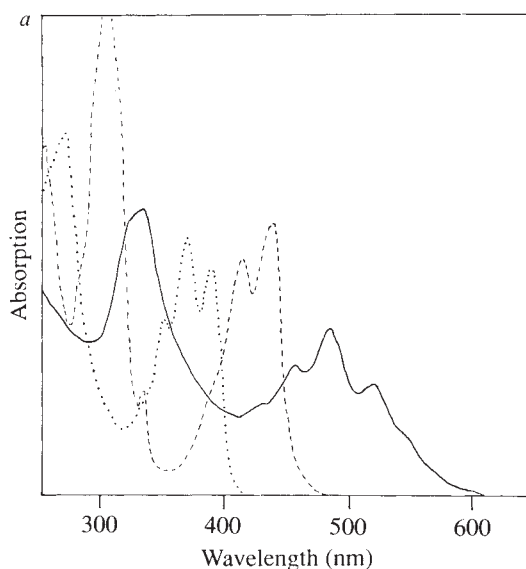
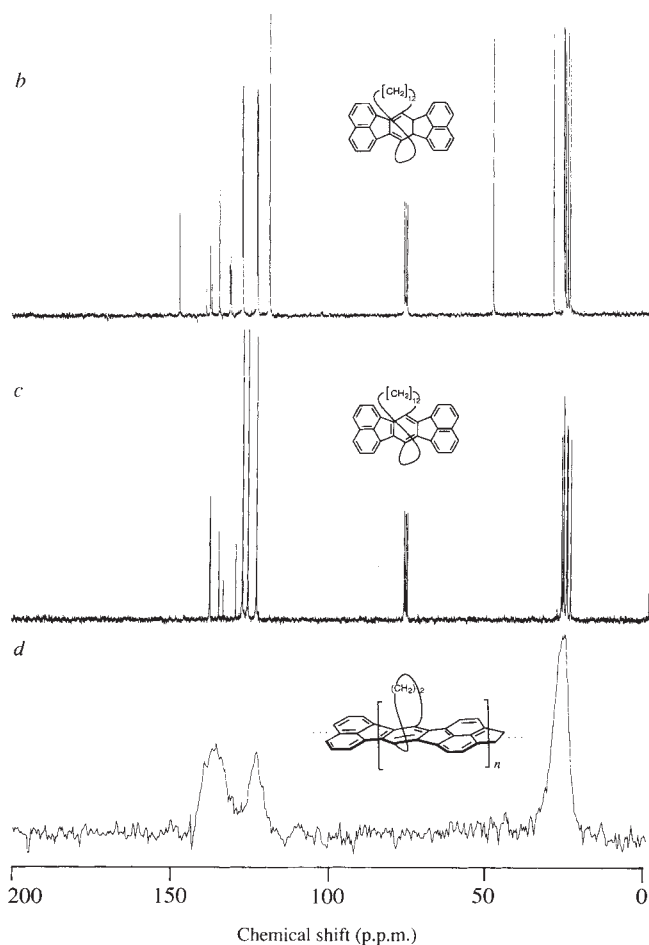


FIG. 5 a, Ultraviolet spectra of compounds **4** (dotted line), **5** (dashed line) and oligomer **1a** (solid line) in chloroform; **4**,  $\log \epsilon$  (441 nm) = 4.385; **5**,  $\log \epsilon$  (389 nm) = 4.038; **1a**,  $\epsilon \approx 0.01$  mM. b, c,  $^{13}C$  NMR spectra of compounds **4** (b) and **5** (c). d, Cross polarization magic-angle spinning (CPMAS)  $^{13}C$  NMR spectrum of polymer **1a**.





To our knowledge, this is the first case where a polymer analogous aromatization reaction of a Diels–Alder ladder polymer can be brought about. The fact that polymer **1a** has an extended area of  $\pi$ -conjugation, is stable toward air and shows a structural relation with ‘unwrapped’ fragments of fullerenes will stimulate various physicochemical and synthetic investigations.  $\square$

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## Growth of oriented molecular sieve crystals on organophosphonate films

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THE successful construction of complex organic/inorganic biomimetic systems<sup>1–3</sup> has demonstrated the great power of supramolecular pre-organization and templating in controlling crystal growth<sup>4</sup>. For instance, polar organic surfaces or surface-attached polar groups can induce the formation of thin films of iron oxide<sup>5</sup>. It would be of great interest, for the design of novel devices such as sensors or catalyst membranes<sup>6</sup>, to be able to control the growth on surfaces of porous crystals with oriented channels: such channels could, for example, control the access of molecules to the surface of a field-effect transistor in a sensor device. Films and membranes with non-oriented channels have been prepared by depositing or growing zeolite<sup>7–12</sup> crystals on metal or metal-oxide supports<sup>13–21</sup>; in one case<sup>21</sup>, pre-grown crystals of an aluminophosphate zeolite were oriented by application of an electric field. Here we report the oriented growth of crystals of a zinc-phosphate zeolite on gold surfaces modified with metal phosphonate multilayer films. We attribute the high degree of orientation (>90%) to a strong affinity between the phosphonic acid groups of the phosphate multilayer and the (111) faces of the growing crystals.

Phosphonic acid monolayers were formed on gold substrates (silicon wafers with a 2,000-Å layer of gold on a 100-Å chromium layer, pre-cleaned with air plasma) by means of sorption of 11-mercapto-1-undecanol (MUD) which was phosphorylated with POCl<sub>3</sub>/2,4,6-collidine<sup>22–24</sup>. The asymmetric and symmetric CH<sub>2</sub> stretching bands for the MUD monolayer (in the reflection-absorption infrared (RAIR) spectrum at 85° incidence) occur at 2,920 and 2,851 cm<sup>-1</sup> (refs 23, 24; 2,919 and 2,850 cm<sup>-1</sup>) both before and after phosphorylation. These frequencies indicate a well-ordered and tightly packed monolayer on the gold surface<sup>25</sup>. In an alternative synthesis, [–S(CH<sub>2</sub>)<sub>4</sub>PO<sub>3</sub>H<sub>2</sub>]<sub>2</sub> (DS)<sup>26</sup> was deposited on gold. The phosphonic acid modified surfaces (layer thick-

ness from ellipsometry: 6–8 Å (DS); 16 Å (MUD)) were immersed sequentially, at 25 °C, in 5.0 mM zirconyl chloride and 1.25 mM 1,10-decanediylbis (phosphonic acid) (DBPA) aqueous solutions to give the zirconium phosphonate (ZrP) trilayer (19 Å thick after the first three steps with [–S(CH<sub>2</sub>)<sub>4</sub>PO<sub>3</sub>H<sub>2</sub>]<sub>2</sub> in the first layer, and 26 Å after three steps with MUD in the first layer). The mid-infrared region of the spectrum of a ZrP trilayer is dominated by the asymmetric phosphonate stretch,  $\nu_a(\text{PO}_3^{2-})$ , at 1,077 cm<sup>-1</sup> (ref. 22; 1,076 cm<sup>-1</sup>). The substrates were rinsed for 30 min with deionized water between all adsorption steps.

The zeolite X analogue (Na, dabco)<sub>96</sub>(ZnPO<sub>4</sub>)<sub>96</sub>·192H<sub>2</sub>O<sup>27</sup> (ZnP) was prepared from a clear solution containing 32 mmol NaOH, 134 mmol 1,4-diazabicyclo[2.2.2]octane (dabco) and 64 mmol H<sub>3</sub>PO<sub>4</sub>, in 75 ml of water that was cooled to 7 °C. To this was added a pre-cooled solution of 48 mmol Zn(NO<sub>3</sub>)<sub>2</sub> in 10 ml of water to give a gel that converts to a ‘milk’ on shaking. The ‘milk’ settles rapidly and the crystallization was completed after 5 h at 7 °C. For thin film depositions, the gold substrates (pre-coated with phosphonate films) were reacted with the above synthesis mixture after the ‘milk’ had just settled. The substrates were carefully placed face-down into the zinc-phosphate mixture for crystallization at 7 °C, for 5 h, then washed with water.

The phosphonate multilayer films promote the nucleation of phosphate molecular sieves from the hydrothermal synthesis mixtures as depicted in Fig. 1. No zeolite crystals become attached to the fresh gold surface without the metal-phosphonate multilayer films. Scanning electron micrographs of the films show that single layers of zinc-phosphate crystals are produced (Fig. 2). The majority (>90%) of the crystals have grown with their basal surfaces oriented parallel to one of the (111) planes of the zeolite structure. They have the same octahedral morphology as the bulk material, with the important difference that the base faces in contact with the substrate are truncated triangles. The more ordered MUD-based ZrP films promote more oriented

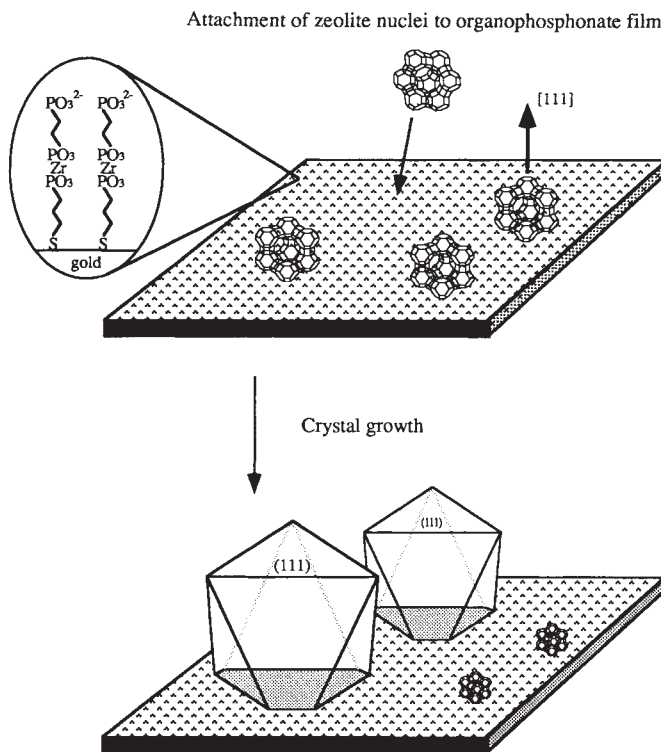


FIG. 1 Proposed reaction scheme for the growth of zinc-phosphate molecular sieves on organophosphonate films.

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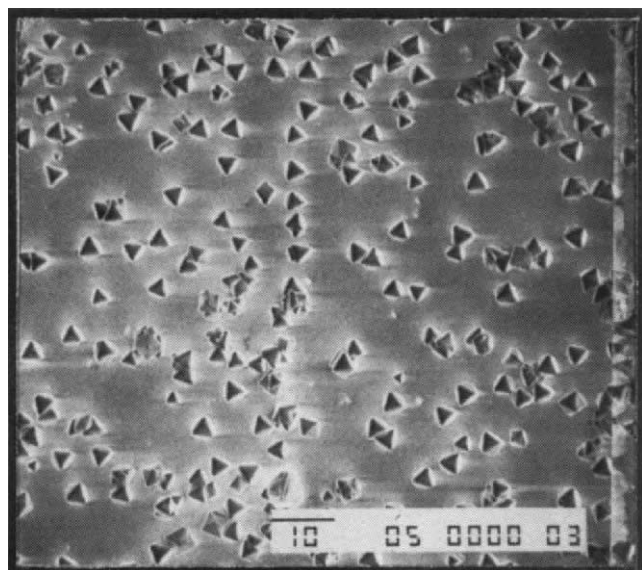


FIG. 2 Scanning electron micrograph of zincophosphate molecular sieves grown on organophosphonate-film-modified gold substrate (scale bar, 10  $\mu\text{m}$ ).

and homogeneous zincophosphate molecular sieve films compared to the  $[-\text{S}(\text{CH}_2)_4\text{PO}_3\text{H}_2]_2$ -based ZrP films.

The almost perfect orientation of the crystals is further supported by the high intensity of the (111) peak (ref. 27) in grazing-angle ( $1.5^\circ$ ) X-ray diffraction patterns (Fig. 3a). The peak positions in the diffraction pattern of the film match perfectly with that of bulk zincophosphate molecular sieve phase as in Fig.

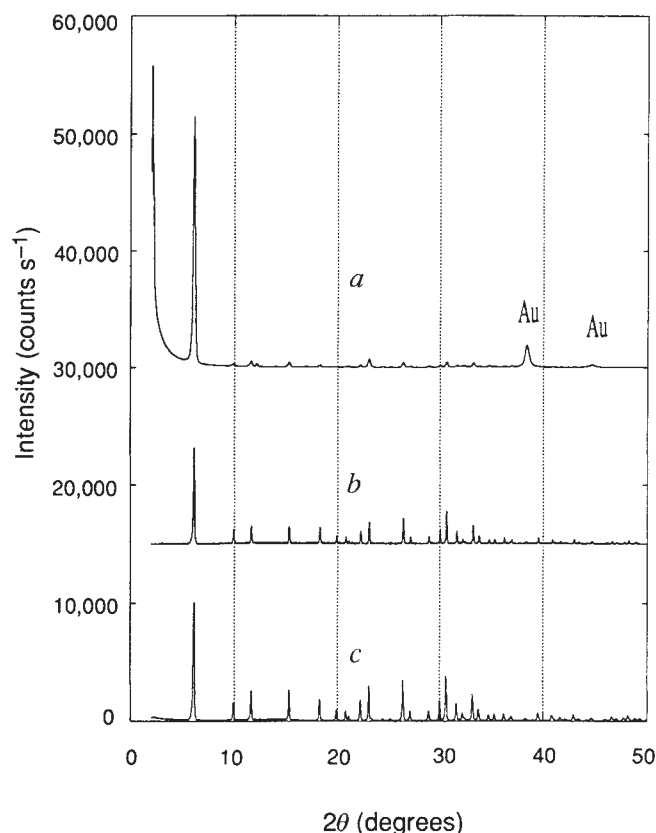


FIG. 3 X-ray diffraction patterns of a, zincophosphate molecular sieve film on organophosphonate films (offset), b, the molecular sieve crystals recovered from the gold substrate (offset,  $2\times$ ) and c, bulk zincophosphate molecular sieve.

3c. In addition, the molecular sieve crystals recovered from the gold substrate gave the same X-ray diffraction pattern and peak ratios as the bulk (Fig. 3b). The zincophosphate crystals are bonded strongly enough to the substrate to survive hours of sonication.

Significant crystal growth occurs only when at least the trilayer MUD(DS)-Zr-DBPA is present on the surface. We associate the absence of crystal growth on the  $-\text{S}(\text{CH}_2)_4\text{PO}_3\text{H}_2$  monolayer with lack of order, and absence of a homogeneous surface of phosphate groups for favourable electrostatic interactions in this film, as indicated by RAIIR spectra. The films obtained after adding zirconyl chloride show no crystal growth at all, suggesting a lack of electrostatic interactions. Finally, the more ordered trilayer MUD(DS)-Zr-DBPA film with phosphonate groups exposed on the outer surface provides an effective nucleation and growth substrate. The organic templating agents are also important to control the molecular sieve growth. No crystal growth occurs when tetramethyl ammonium hydroxide (TMA) instead of dabco is used as the template. This effect may be related to the dipositive charge on dabco and its resulting surface association in the synthesis.

What is the reason for the dramatic effect of the organic layers on the crystal growth? Quenching reactions were used to explore the association between the phosphonate surface and zeolite crystal faces. To the bulk zincophosphate crystallization mixture (with 57.6 mmol  $\text{H}_3\text{PO}_4$ ) was added methylphosphonic acid (6.4 mmol  $\text{H}_2\text{PO}_3\text{CH}_3$ ) after 30 min and after 1 h, and the mixture was kept at  $7^\circ\text{C}$  for another 5 h. If the phosphonic acid derivative attaches to the crystal surface, the tail group ( $-\text{CH}_3$ ) could be expected to block further crystal growth. X-ray diffraction patterns and electron micrographs show that addition of the 'quenching agent' stops crystallization immediately. The results confirm that the phosphonic acid groups have a strong affinity to the growing (111) faces of the molecular sieve crystals.

To explore the porosity of the zincophosphate films, the occluded dabco molecules were exchanged with cations to avoid the conventional high temperature calcination treatment which is known to destroy the zincophosphate structure<sup>27</sup>. ZnP film was grown on a quartz crystal microbalance<sup>20,28</sup> with gold electrodes, as described above, and treated with 0.5 M NaCl solution for 12 h (the removal of dabco in similar bulk experiments is fairly complete; the carbon content decreases from 24 to 2.4 wt%). The film was then dehydrated in a stream of dry helium (12 h at  $20^\circ\text{C}$ ), and its water sorption isotherm was

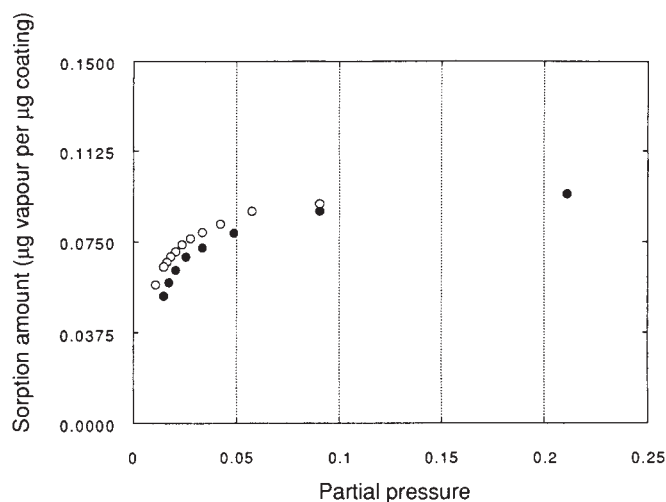


FIG. 4 Water sorption isotherm of zincophosphate crystals grown on the surface of a quartz crystal microbalance at  $20^\circ\text{C}$  (partial pressure is  $P/P_0$ , and  $P_0=17.5$  torr). Vapour exposure was  $\sim 12$  min per data point. The small offset of the desorption points shows that the film was close to equilibrium, even at this low temperature.



measured *in situ* in a flow system (Fig. 4). The typical micropore sorption isotherm (here with saturation at a partial pressure  $P/P_0$  of  $\sim 0.09$ ) is apparent and confirms the presence of molecular sieve material in the film.

In conclusion, we have discovered strategies to grow oriented molecular sieve crystals on gold substrates. Zirconium phosphonate surfaces most probably interact with the growing inorganic zincophosphate crystals through electrostatic and geometric matching processes. The  $[-\text{PO}_3\text{H}_2]$  surfaces are effective at stabilizing the  $\{111\}$  faces of zincophosphate. It seems unlikely that individual  $\text{Zn}^{2+}$  and  $\text{PO}_4^{3-}$  ions can assemble the zeolite structure on the organic film. We propose that small

zincophosphate nuclei attach and orient themselves on the film, possibly assisted by preadsorption of  $\text{Zn}^{2+}$  or dabco species on the surface (Fig. 1).

To our knowledge, these systems are the first examples of oriented, surface-controlled growth of molecular sieve crystals. Further studies of the surface reactions involved here should increase our understanding of nucleation processes in molecular sieve synthesis, and additional organized systems should result. These materials could offer exciting applications, such as controlled access of molecules of pre-selected size to a sensor surface, or orientation of molecules for nonlinear optical applications. □

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## Formation of amide bonds without a condensation agent and implications for origin of life

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AMIDE bonds are of central importance for biochemistry; in the guise of peptide bonds, they form the backbone of proteins. The formation of amide bonds without the assistance of enzymes poses a major challenge for theories of the origin of life. Enzyme-free formation of amide bonds between amino acids has been demonstrated in the presence of condensing agents such as cyanamide<sup>1–4</sup>. Here we report the formation of amide bonds in aqueous solution in the absence of any condensing agent. We find that the formation of pyrite ( $\text{FeS}_2$ ) from  $\text{FeS}$  and  $\text{H}_2\text{S}$  can provide the driving force for reductive acetylation of amino acids with mercaptoacetic acid ( $\text{HSCH}_2\text{COOH}$ ). The redox energy of pyrite formation permits the activation of the carboxylic acid group, which is converted to a species that reacts readily with amines. This process provides support for the chemo-autotrophic theory<sup>5–8</sup> for the origin of life, in which pyrite formation supplies the energy source for the first autocatalytic reproduction cycle.

The biosphere is divided between heterotrophic and autotrophic ways of life, raising the question of whether life began with one or the other. It is widely thought that life began heterotrophically in a prebiotic broth of nucleotides (the RNA world<sup>9,10</sup>) and/or amino acids (for review see ref. 11). These theories suffer from the lack of a geochemically plausible energy source for driving the postulated condensation reactions to

nucleic acids and/or polypeptides in an aqueous environment.

The theory of a chemo-autotrophic origin postulates as the first energy source for life the oxidative formation of pyrite from iron sulphide and hydrogen sulphide ( $\text{FeS} + \text{HS}^- \rightarrow \text{FeS}_2 + \text{H}^+ + 2\text{e}^-$ ;  $E^0 = -620 \text{ mV}$ )<sup>5,6</sup>. The electrons are seen as being available for a reductive carbon-dioxide fixation cycle, specifically an archaic autocatalytic version of the reductive citric-acid cycle<sup>7</sup>. Within this theory  $\alpha$ -keto acids play a central role. It has been postulated that they can be reduced at the  $\alpha$ -position by the addition of hydrogen sulphide and subsequent desulphurization

TABLE 1 Acetylation of amino compounds

| Amine<br>(100 $\mu\text{mol}$ ) | $\text{HSCH}_2\text{COOH}$<br>( $\mu\text{mol}$ ) | $\text{H}_2\text{S}$<br>( $\mu\text{mol}$ ) | <i>N</i> -acetyl compounds ( $\mu\text{mol}$ ) |      |
|---------------------------------|---------------------------------------------------|---------------------------------------------|------------------------------------------------|------|
|                                 |                                                   |                                             | 2 d                                            | 4 d  |
| Aniline                         | 50                                                | 2,000                                       | 0.55                                           | 0.9  |
| Aniline                         | 50                                                | 0                                           | 0.25                                           | 0.4  |
| <i>o</i> -Carboxy-aniline       | 50                                                | 2,000                                       | 0.29                                           | 0.42 |
| Tyrosine                        | 5,000                                             | 2,000                                       | 1.2                                            | 1.8  |
| Tyrosine                        | 5,000                                             | 0                                           | 0.2                                            | 0.3  |

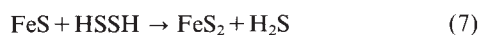
All procedures were carried out under argon. The solutions were prepared from doubly distilled water through which Ar had been bubbled for 2 h. Amorphous  $\text{FeS}$  was prepared in an anaerobic chamber by adding a solution of  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  to 0.6 M  $\text{FeSO}_4$ , filtering the precipitate, washing it with  $\text{H}_2\text{O}$  and drying it under  $\text{N}_2$ : $\text{H}_2 = 95:5$ . The  $\text{H}_2\text{S}$  was prepared by adding 50%  $\text{H}_2\text{SO}_4$  to  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  in an evacuated serum bottle. Mercaptoacetic acid (Merck) was distilled under vacuum and immediately dissolved in water to a concentration of 5 mM. In the anaerobic chamber, serum bottles (120 ml) were charged with 10 ml  $\text{H}_2\text{O}$ , 2 mM  $\text{FeS}$ , 100  $\mu\text{M}$  of the amino compound and an amount of mercaptoacetic acid given in the Table, closed with a 'viton' diaphragm (Ochs), supplied with an Ar atmosphere (200 kPa) and subsequently charged with  $\text{H}_2\text{S}$  (2 mmol). The pH was adjusted to 4.5. All incubations were carried out at 100 °C in a rotary shaker (100 r.p.m.). The yield of the *N*-acetyl compound ( $\mu\text{mol}$ ) was determined after 2 and 4 days by high-performance liquid chromatography (HPLC) under the conditions given in Fig. 1. It decreases with increasing pH (not shown).

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with FeS:



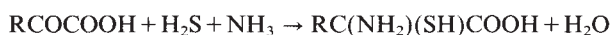
It has further been postulated<sup>8</sup> that this desulphurization proceeds through an  $\alpha$ -carbanion intermediate. This would mean that the reaction rate can be increased by a resonance stabilization of the  $\alpha$ -carbanion. The free carboxylate group ( $-\text{COO}^-$ ) is not available for such resonance. Therefore, it was postulated that a pre-equilibrium would convert the carboxylate group into an activated derivative capable of the required resonance. This proposal is shown in reactions (4) to (8) for the case of mercaptoacetic acid activated by the formation of a  $-\text{COSH}$  group:



This proposal means that the reaction sequence (4) to (8) proceeds at a much higher rate than the direct desulphurization (3). Therefore, the entire redox flow would be channelled through thioacetic acid ( $\text{CH}_3\text{COSH}$ ). Reaction (7) provides the thermodynamic pull. Reactions (5) to (7) are seen as occurring on the surface of growing pyrite crystals, perhaps by way of iron-sulphur clusters<sup>6,8</sup>. By this mechanism, an exergonic redox reaction in one part of the organic molecule ( $\text{HSCH}_2^- \rightarrow \text{CH}_3^-$ ) is coupled with an endergonic group activation reaction in another part of the same molecule ( $-\text{COOH} \rightarrow -\text{COSH}$ ). The resulting thioacetic acid should be available for a variety of synthetic reactions, notably aminolysis, reaction (9), in competition with hydrolysis, reaction (8).



It has been proposed<sup>8</sup> that by a mechanism similar to that shown in reactions (4) to (7),  $\alpha$ -ketoacids may be reductively aminated to activated amino acids:



The activated amino acids may subsequently undergo condensation into surface-bound peptides.

The results described here provide support for these proposals. Specifically, we consider the formation of acetamides by reactions (4) to (9). All experiments have been carried out under fastidiously anaerobic conditions. We first chose aniline as a trapping agent in reaction (9), because it is available as a free base over a wide pH range. It was reacted at pH 4.5 with mercaptoacetic acid and FeS in the presence or absence of added  $\text{H}_2\text{S}$ . High-performance liquid chromatography (Fig. 1; Table 1), gas chromatography and thin-layer chromatography revealed that a new organic product had been formed in significant amounts. At the same time, the reaction mixture showed a glistening surface which is characteristic of anaerobic pyrite formation<sup>12</sup>. The product was identified by mass spectroscopy as acetanilide (Fig. 2). The proposed mechanism of the novel reaction is supported by the finding that the formation of acetanilide is promoted by the addition of  $\text{H}_2\text{S}$ . The formation of acetanilide without added  $\text{H}_2\text{S}$  is attributed to a dissolution of iron sulphide ( $\text{FeS} + \text{H}^+ \rightarrow \text{Fe}^{2+} + \text{HS}^-$ ). In the absence of FeS, no acetanilide is formed, even if  $\text{H}_2\text{S}$  is added. Moreover, under the same reaction conditions, in the presence of FeS and  $\text{H}_2\text{S}$ , the formation of acetanilide from acetic acid and aniline is not detectable and neither is its hydrolysis. This excludes the possibility that

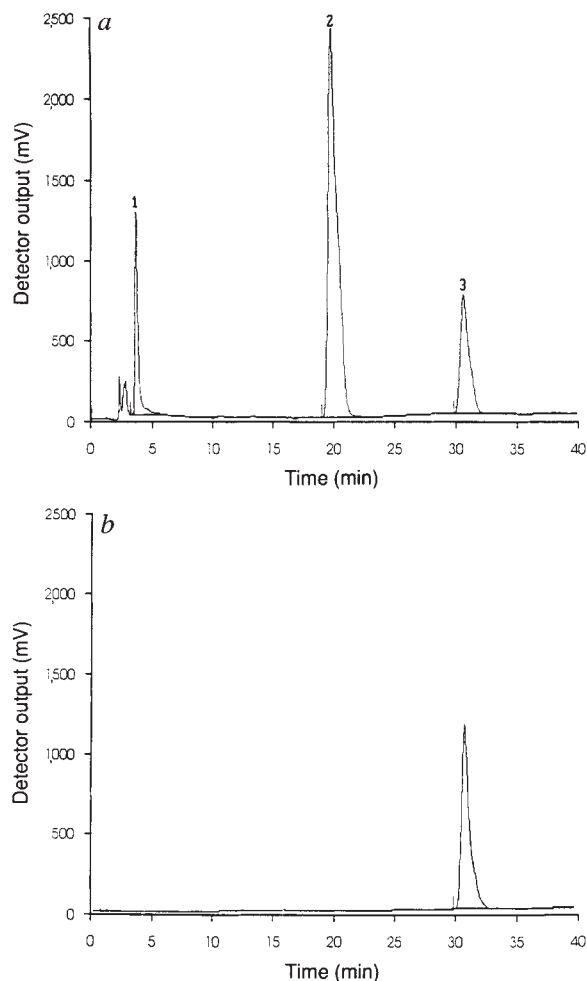


FIG. 1 Identification of acetanilide by high-performance liquid chromatography (HPLC). a, Chromatogram of reaction mixture with FeS/ $\text{H}_2\text{S}$  (Table 1). Peaks 1, 2 and 3 show  $\text{H}_2\text{S}$ , aniline and acetanilide respectively. b, Chromatogram of acetanilide standard (Aldrich).

METHODS. A portion of the reaction mixture (Table 1 legend) was centrifuged and 20  $\mu\text{l}$  supernatant was loaded onto a C-18 reversed-phase HPLC column (Kontron) in 0.05 M phosphate buffer (pH 7.0) and separated by gradient steps: 5% methanol (6 min), 5–10% methanol (6–8 min), 10–15% methanol (8–12 min), 15% methanol (12–20 min), 15–40% methanol (20–40 min). Flow rate was 1  $\text{ml min}^{-1}$ . Aromatic compounds were detected by monitoring the absorbance at 254 nm.

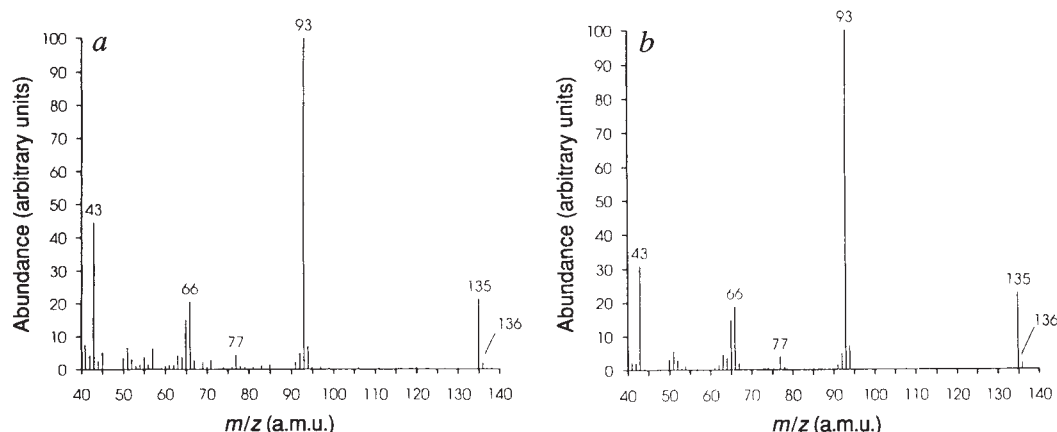
acetanilide is formed as an equilibration product. Moreover, the possibility of a competition between aminolysis, reaction (8), and hydrolysis, reaction (9), was demonstrated by the formation of acetanilide in a diluted aqueous solution of aniline and thioacetic acid under the chosen reaction conditions. Reaction (7) is well known and the formation of pyrite from FeS/ $\text{H}_2\text{S}$  has been previously demonstrated<sup>12</sup>. Also, the catalytic role of  $\text{H}_2\text{S}$  in the formation of acetic acid from aqueous mercaptoacetic acid and FeS/ $\text{H}_2\text{S}$  was observed earlier<sup>13</sup>. It should be noted that under the reaction conditions thioacetic acid is a short-lived intermediate. Therefore, its direct detection is not possible.

Turning then to the acetylation of amino acids, we replaced aniline by *o*-carboxy-aniline. With FeS/ $\text{H}_2\text{S}$  it gave rise to *o*-carboxy-acetanilide (Table 1). This means that the carboxy group lowers the yield without inhibiting the reaction. Next, we tested for the acetylation of tyrosine and found that *N*-acetyl-tyrosine was indeed formed, as detected by high-performance liquid chromatography (Table 1). In view of the high  $\text{pK}_a$  of tyrosine compared to aniline (9.11 compared to 4.64), and the pH of 4.5, an excess concentration of mercaptoacetic acid was chosen.



FIG. 2 Mass spectra of acetanilide. *a*, Reaction mixture with FeS/H<sub>2</sub>S (Table 1). *b*, Acetanilide standard (Aldrich).

**METHODS.** The reaction mixture was centrifuged and the supernatant extracted with chloroform. The extract was purified by thin-layer chromatography (in ethyl acetate:hexane, 1:1). The product was scraped off the thin-layer plate and extracted with chloroform. The mass spectrum was taken with a Finnigan MAT 112 S (electron ionization; 70 eV, direct insert).



Our finding demonstrates the formation of amide bonds from free -COOH and -NH<sub>2</sub> groups in a highly dilute, hot, aqueous solution without an enzyme and without a condensation agent. It should be compared with the amide formation by the oxidation of a Schiff base with hydrogen peroxide which has been suggested as a prebiotic reaction<sup>14</sup> and which may be considered as the closest previous approach to this reaction. By a similar reaction a thioester has been produced from glyceraldehyde and a mercapto compound, either in a redox reaction with oxygen or by rearrangement<sup>15</sup>. DeDuve<sup>11</sup> has stressed the importance of thioesters for an early metabolism. He has suggested that thioesters are formed by the equilibration of mercaptanes with free acids in a prebiotic broth at high temperatures, and that these thioesters may constitute the first energy source of life. By contrast, the thioacid activation proposed here is seen as an intermediate within an irreversible redox energy flow into the thermodynamic sink of pyrite. Our result goes a long way in demonstrating the potential role of pyrite chemistry in a chemotrophic origin of life. □

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## Soil acidification and nitrogen saturation from weathering of ammonium-bearing rock

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**THE origin of small regions of extremely acidic (pH < 4.5) soils in the Klamath mountains of northern California has long been a mystery. These acidic regions are devoid of coniferous vegetation, although surrounded by healthy coniferous forest. Here we show that the extreme soil acidification is caused by nitrogen inputs from ammonium-containing bedrock. Analyses of soil solution composition and bedrock mineralogy reveal that oxidation of ammonium released from the mica schist bedrock generates high levels of nitric acid. The consequent acidity mobilizes potentially toxic levels of aluminium and causes intense leaching of nutrient cations. In the adjacent healthy forest, plant uptake of nitrate attenuates these effects. We suggest that a natural perturbation (for example a small forest fire) caused initial loss of vegetation from the barren regions. We also suggest that subsequent erosion led to serious nutrient depletion of these soils, and that extreme acidification, potentially toxic levels of aqueous aluminium and nutrient deficiencies resulting from cation leaching played a significant role in preventing regrowth. These results show that geo-**

**logical nitrogen, commonly overlooked in biogeochemical cycling but known to be present in appreciable quantities in certain rocks<sup>1–8</sup>, may represent a large and reactive pool which can have significant ecological effects.**

Geological nitrogen has been found in sedimentary, metamorphic and altered igneous rocks in a wide variety of environments<sup>1–8</sup>. It is particularly common in sedimentary and

TABLE 1 Composition of mica schist

| Compound                          | Concentration (%) | Standard deviation (%) |
|-----------------------------------|-------------------|------------------------|
| SiO <sub>2</sub>                  | 58.15             | 2.81                   |
| TiO <sub>2</sub>                  | 0.97              | 0.08                   |
| Al <sub>2</sub> O <sub>3</sub>    | 21.34             | 1.46                   |
| Cr <sub>2</sub> O <sub>3</sub>    | 0.08              | 0.05                   |
| FeO                               | 9.20              | 0.92                   |
| MgO                               | 4.95              | 0.44                   |
| CaO                               | 0.29              | 0.05                   |
| MnO                               | 0.08              | 0.04                   |
| Na <sub>2</sub> O                 | 2.54              | 0.18                   |
| K <sub>2</sub> O                  | 1.93              | 0.10                   |
| (NH <sub>4</sub> ) <sub>2</sub> O | 0.52              | 0.04                   |
| Total                             | 100.05            |                        |
| Organic C                         | 0.41*             |                        |

These chemical analyses are the mean of six samples of unweathered mica schist (South Fork Mountain Schist) bedrock. Analysis after HF digestion, reported on an ignited weight basis.

\* Determined by combustion.

TABLE 2 Selected chemical properties of forest soil and barren-area soil

| Property                                 | Forest soil |      |      |      |      | Barren soil |      |      |      |
|------------------------------------------|-------------|------|------|------|------|-------------|------|------|------|
| Soil horizon                             | FF          | A    | AB   | Bw   | BC   | A           | AB   | Bw   | BC   |
| pH                                       | 4.63        | 4.80 | 4.63 | 4.75 | 4.76 | 4.31        | 4.32 | 4.45 | 4.46 |
| Exchangeable cations                     |             |      |      |      |      |             |      |      |      |
| Ca (cmol <sub>c</sub> kg <sup>-1</sup> ) | 10.9        | 3.9  | 0.1  | 0.1  | 0.1  | <0.1        | <0.1 | <0.1 | <0.1 |
| Mg                                       | 6.1         | 1.5  | 0.1  | 0.1  | 0.1  | <0.1        | <0.1 | 0.1  | 0.1  |
| K                                        | 1.6         | 0.4  | 0.2  | 0.2  | 0.1  | 0.1         | 0.1  | 0.1  | 0.1  |
| Na                                       | 0.6         | <0.1 | <0.1 | <0.1 | <0.1 | <0.1        | <0.1 | <0.1 | <0.1 |
| Al                                       | 0.1         | 4.1  | 4.3  | 4.6  | 3.9  | 4.1         | 4.5  | 4.1  | 3.9  |
| Base saturation (%)                      | 97.6        | 46.9 | 6.8  | 4.6  | 3.8  | 3.7         | 3.0  | 4.3  | 4.8  |
| Organic carbon (g kg <sup>-1</sup> )     | 320         | 157  | 36   | 33   | 31   | 40          | 35   | 25   | 23   |
| Nitrogen (g kg <sup>-1</sup> )           | 24.4        | 18.7 | 9.2  | 8.0  | 5.6  | 8.6         | 8.8  | 6.5  | 5.7  |
| C/N ratio                                | 13.1        | 8.4  | 3.9  | 4.1  | 5.6  | 4.7         | 4.0  | 3.9  | 4.0  |

Results for each type of soil represent the mean of six soil samples. Analysis methods as follows: pH, 1:1 soil:water; exchangeable cations, 1M NH<sub>4</sub>Cl extract; organic carbon, dry combustion with Leco carbon analyser; nitrogen, modified Kjeldahl digestion<sup>12</sup>. (cmol<sub>c</sub> kg<sup>-1</sup> indicates cmol of charge per kg of soil.)

metasedimentary rocks, the former being the dominant rock type on the Earth's surface. But standard analytical methods for elemental analysis of rocks and minerals do not determine nitrogen content, and so its presence and potential importance are often overlooked.

South Fork Mountain schist forms a narrow linear belt several hundred kilometres in length along the boundary fault separating the Klamath mountains and the Coast Range provinces in northern California and southern Oregon<sup>9-10</sup>. Within this belt, numerous areas up to 20 ha in size and devoid of coniferous vegetation are found interspersed in healthy mixed coniferous forests. The only vegetation existing on these barren areas is a scattering of the prostrate herb, *Calyptridium umbellatum* ('pussy paws'). Soil characterization indicated that the soils comprising the barren area were extremely acidic but no explanation was apparent to reveal the source of their acidity.

The South Fork Mountain schist has a mineralogical assemblage consisting of quartz-albite-mica-chlorite<sup>11</sup> that formed from low-grade metamorphism of carbonaceous, argillaceous mudstone<sup>9-11</sup>. During diagenesis, nitrogen is released from organic matter and converted to ammonium<sup>5,7,8</sup>. Ammonium forms a solid solution with potassium in the interlayer position of 2:1 clay minerals (for example illite, illite/smectite)<sup>4</sup>, the amount of ammonium incorporation being regulated by the nitrogen content of the original sediments<sup>8</sup>. The mica schist examined in this study contained 0.27% N (0.52% (NH<sub>4</sub>)<sub>2</sub>O) (Table 1), corresponding to 7.1 Mg ha<sup>-1</sup> of nitrogen contained within each 10-cm thickness of the bedrock. Leaching ground rock material with MgCl<sub>2</sub> confirmed that ammonium, rather than nitrate, was the principal form of nitrogen in the rock.

The nitrogen content of BC horizons (weakly weathered parent material) in the forest and barren-area soils is similar, indicating that spatial variability in bedrock nitrogen content is not responsible for localized patches of extreme acidification (Table 2). Chemical analysis also showed a very high magnesium content relative to calcium (Mg/Ca atomic ratio is 24) leading to the potential for a nutrient imbalance similar to those found in serpentine parent materials.

The reason for the initial loss of forest vegetation from the barren areas is unknown, but is believed to be related to natural perturbations, such as small forest fires associated with lightning strikes. Evidence for historic coniferous vegetation on the barren-area soils results from remnants of highly decomposed tree roots in the barren-area soils and comparable levels of organic carbon (~30 g kg<sup>-1</sup>) in subsoil horizons of the forest soil and the truncated barren-area soil (Table 2). Loss of forest vegetation has contributed to moderate-to-severe erosion and loss of the 15-cm-thick litter layer common on the forest soils. A comparison of solid-phase properties between the two sites shows that the forest soil has a greater pH (0.3–0.5 units higher) and a higher base saturation in the upper soil horizons (Table 2). The higher exchangeable cation concentrations in the forest soil are maintained by biocycling. Carbon/nitrogen ratios of modified Kjeldahl digests<sup>12</sup> were <10 for mineral horizons in both soils, reflecting the contribution of geological nitrogen.

The source of the extreme soil acidity in the barren area soil was revealed by analysis of soil solutions extracted by centrifugation<sup>13</sup>. Nitrate was the dominant anion (>87%) and was electrically balanced primarily by H<sup>+</sup> and aluminium (Fig. 1). Concentrations of the essential nutrients calcium, magnesium

FIG. 1 Soil solution chemistry from forest soil (top) and barren-area soil (bottom) collected by centrifugation during the autumn of 1991. Concentrations of anions and cations in the charge balance diagram are equal to the width of the compartment. Organic anion contribution was taken as the difference between the summation of measured inorganic cations and anions<sup>19</sup>. The order of elements shown from left to right are anions: organic anion, NO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and cations: K<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, Mg<sup>2+</sup>, Ca<sup>2+</sup>, Al<sup>3+</sup>, H<sup>+</sup>. Major cations and anions were determined by ion chromatography. Aqueous aluminium was fractionated by the automated pyrocatechol violet method<sup>26</sup> into strongly bound Al, non-labile monomeric Al (primarily organic complexes) and labile monomeric Al (primarily inorganic complexes with F<sup>-</sup>, SO<sub>4</sub><sup>2-</sup> and OH<sup>-</sup>)<sup>15</sup>. All values represent the mean of six samples. (μmol<sub>c</sub> l<sup>-1</sup> indicates μmol of charge per l.)

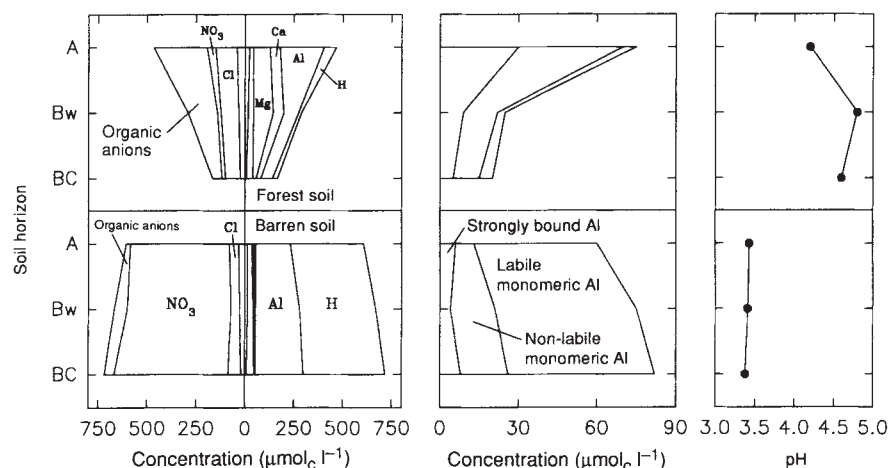
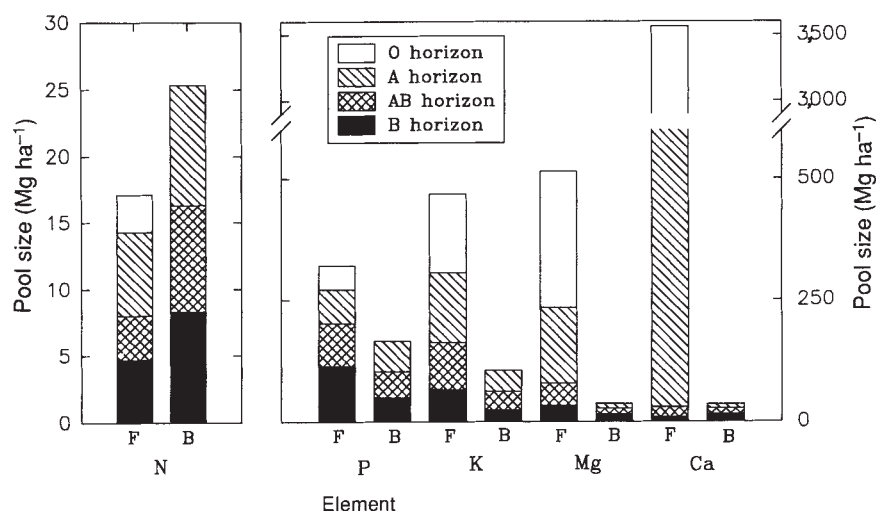


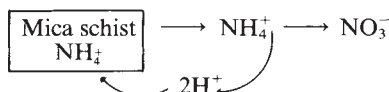


FIG. 2 Elemental pools of nitrogen and phosphorus determined by modified Kjeldahl digestions and pools of exchangeable potassium, magnesium and calcium displayed by horizon for the forest soil (F) and barren-area soil (B). All values represent the mean of six samples.



and potassium were very low ( $<10 \mu\text{M}$ ) indicating a strong potential for deficiencies of these nutrients. The pH values of soil solution in the barren-area soil were about one unit lower than comparable horizons in the forest soil. At pH values below 4.5, dissolution of aluminium is the primary acid-neutralizing reaction<sup>14</sup>. The high concentration of labile-monomeric aluminium<sup>15</sup> (primarily inorganic forms of Al) in the barren-area soil indicates a strong potential for aluminium toxicity<sup>16,17</sup>. In contrast, most of the dissolved aluminium in the forest soil is complexed with organic acids, rendering the aluminium non-toxic<sup>18</sup>. The abundance of organic acids in the forest soil is indicated by the large anion-charge deficit originating from dissociated organic acids leaching from the forest canopy and litter layer<sup>19</sup>.

Concentrations of ammonium in soil solutions were less than  $10 \mu\text{M}$ , indicating nearly complete conversion of ammonium to nitrate by the nitrification process. This provides additional evidence for active nitrification under highly acidic conditions in forest soils<sup>20</sup>. The nitrification process results in positive feedback to the weathering process by releasing protons, which enhance mineral weathering rates leading to further release of ammonium<sup>21</sup>.



Laboratory incubations<sup>22</sup> measured nitrification rates of 39 mg N per kg soil over a 2-week period for the surface mineral horizons (A horizon) of both barren-area and forest soils. These incubations indicate that proton production by nitrification was similar for both soils; however, plant uptake of nitrate in the forest consumes protons either by simultaneous uptake of  $\text{H}^+$  or release of bicarbonate or organic alkalinity to maintain charge balance within the plant<sup>14</sup>. The net nitrogen retention capacity of coniferous forest is generally in the range  $5\text{--}30 \text{ kg ha}^{-1} \text{ yr}^{-1}$  (ref. 23). As long as nitrogen retention equals nitrogen release rates from geological material, no soil acidification occurs from the nitrification process. Thus, plant uptake of nitrate is an important mechanism for attenuating soil acidification in the forest soil<sup>24,25</sup>. Plants further conserve nutrients by biocycling cations, such as calcium, magnesium, and potassium, thereby maintaining a higher base saturation and pH.

Maintenance of cation nutrient pools by biocycling is clearly illustrated by comparing nutrient pools between barren-area and forest soils (Fig. 2). The pools of nutrient cations are strongly depleted in the barren-area soils. The major source of these cations is from the litter layer and the A horizon; soil layers which were largely removed from barren-area soils by erosion and/or fires. This demonstrates the importance of conserving

the litter layer and humus-rich A horizon for maintaining the nutrient status of this ecosystem. The lower nitrogen pool in the forest soil is probably due to a more advanced stage of weathering in the upper soil horizons, leading to a depletion of ammonium in the parent material. In contrast, erosion of the weathered surface horizons from the barren-area soil has exposed relatively fresh geological material that has not been depleted of its geological nitrogen.

This study shows that the geological nitrogen in this ecosystem is a large and reactive pool that has the potential to cause extreme soil acidification. Removal of vegetation in this ecosystem decouples the intricate biogeochemical cycle leading to soil acidification, loss of nutrient pools through erosion and leaching, and potentially toxic concentrations of inorganic aluminium. This example of nitrogen saturation may serve as a natural analogue for examining the potentially devastating effects of anthropogenic induced nitrogen saturation, such as those occurring from atmospheric nitrogen deposition, excessive use of ammonium fertilizers, and timber harvest. Extreme care must be exercised in forest management practices in these sensitive ecosystems to preserve the natural nutrient cycle that attenuates soil acidification and maintains the nutrient status. □

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## $^{210}\text{Po}$ – $^{210}\text{Pb}$ dating of recent volcanic eruptions on the sea floor

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THE globe-encircling mid-ocean ridge system accounts for most of the Earth's volcanism and influences its heat budget, morphology and the composition of the oceans. It is therefore important to constrain the size, frequency and compositional variability of ridge eruptions, but this is made difficult by the remoteness of much of the sea floor and the paucity of applicable radioactive chronometers. Remote monitoring has provided indirect evidence for active ridge volcanism<sup>1–3</sup>, which in some cases has been strengthened by submersible observations<sup>4,5</sup>, but it has not been possible to date these eruptions directly. Here we present a new chronometer based on  $^{210}\text{Po}$ – $^{210}\text{Pb}$  radioactive disequilibrium, which allows us to date glassy eruption products within a few years of their eruption. Our first results, on lavas from 9° 50' N on the East Pacific Rise, confirm that the ridge erupted only months before sample collec-

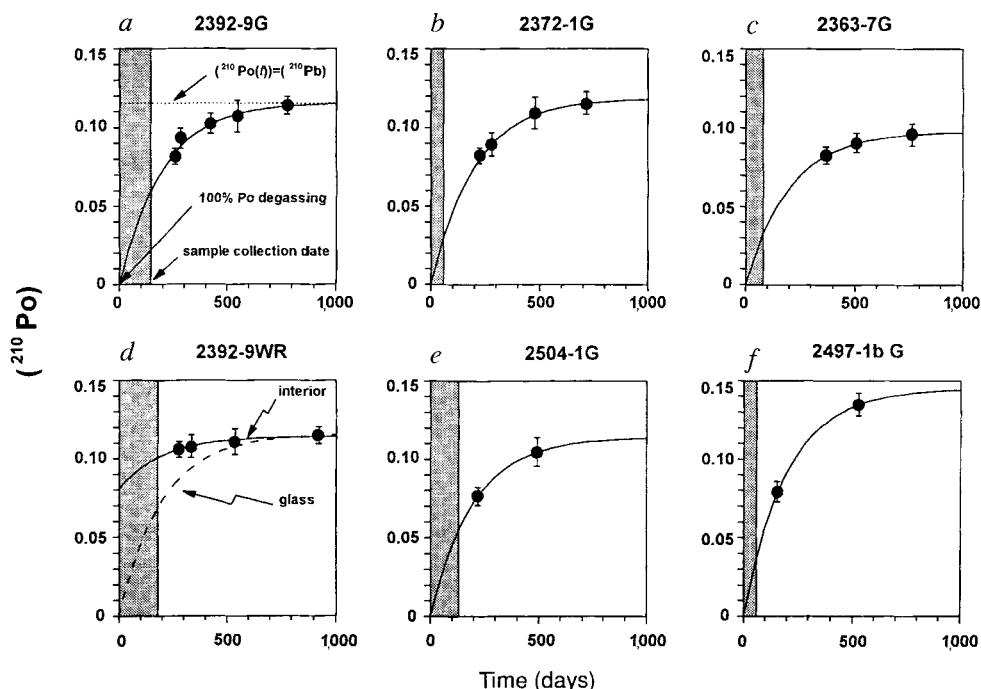
tion, and indicate that the eruptions extended over at least one year. The chronometer begins with Po volatilization during eruption (all glasses analysed were 75–100% degassed). A crystalline pillow interior was <20% degassed, however, implying that the flow surface may control Po degassing during deep submarine eruptions. Although the  $^{210}\text{Po}$ – $^{210}\text{Pb}$  chronometer is useful for only a short time-frame, it should prove valuable for dating recent but unobserved submarine eruptions and determining details of eruptive sequences.

$^{210}\text{Po}$  is a member of the  $^{238}\text{U}$  decay chain and has the same activity as all other nuclides in the chain in an undisturbed system (that is, at secular, or radioactive, equilibrium). Polonium is, however, moderately volatile and in volcanic emissions is completely gaseous at ~400 °C (ref. 6). It has been suggested that Po volatilizes as a halogen-containing species<sup>7,8</sup>, which is reasonable considering that the di- and tetra-halogen compounds of Po boil or sublime at temperatures between 110 and 390 °C (ref. 9). In newly erupted subaerial lavas at Kilauea, Mount St Helens and Etna volcanoes<sup>8,10,11</sup> and in the shallow submarine summit of Macdonald seamount<sup>12</sup>,  $^{210}\text{Po}$  is essentially completely (>95%) degassed. In such lavas the  $^{210}\text{Po}$  activity grows back toward equilibrium with its parents in the decay chain following its half-life of 138.4 d. Although  $^{210}\text{Po}$  is the immediate daughter of  $^{210}\text{Bi}$ , the latter has a very short half-life (~5 d) and after a fractionation event, quickly comes to equilibrium with its parent,  $^{210}\text{Pb}$ . The rate of change of  $^{210}\text{Po}$  in newly erupted lavas is therefore effectively governed by  $^{210}\text{Pb}$  decay within 25–30 d after eruption. In contrast to  $^{210}\text{Po}$ , little or no  $^{210}\text{Pb}$  degasses (<1%) during eruption, based on high  $^{210}\text{Po}/^{210}\text{Pb}$  ratios in volcanic plume materials<sup>7,13–17</sup>. Thus degassing of  $^{210}\text{Po}$  sets the  $^{210}\text{Po}$ – $^{210}\text{Pb}$  clock for volcanic rocks during eruption, and time since eruption is determined by the extent of ingrowth to equilibrium with  $^{210}\text{Pb}$ .

The  $^{210}\text{Po}$  content of degassed lavas as a function of time is described by a simplified form of the Bateman equation for closed-system decay:

$$(^{210}\text{Po})_t = (^{210}\text{Po})_{t=0} \times [e^{-\lambda t}] + (^{210}\text{Pb}) \times [1 - e^{-\lambda t}]$$

FIG. 1 a–f,  $^{210}\text{Po}$  ingrowth curves and eruption windows for basalts analysed in this study. ( $^{210}\text{Po}$ ) indicates the activity of this species (in d.p.m. g<sup>-1</sup>). All samples except 2392-WR are from glassy flow margins; note the different degassing behaviours of glass and crystalline flow interior for this sample (d). Ingrowth curves were fitted to the data using the Levenberg–Marquardt method (iterative, non-linear least-squares minimization of x and y axis residuals)<sup>25</sup>. The timescale is fixed by the decay constant and the known intervals between analyses. The position of the curve within the data is fixed, as is the asymptote; the initial  $^{210}\text{Po}$  activity is constrained to lie on this curve. Time t is set to 0 at ( $^{210}\text{Po}$ ) = 0 to determine the eruption time if degassing is complete at eruption; this corresponds to the maximum age of the lava flow, because, as discussed in the text, degassing may be incomplete. The quality of curve fit is highest if there are many analyses spread throughout the grow-in period. Thus the 'regressions' for samples 2504-1G and 2497-1bG are less robust than others because there are no degrees of freedom in the solution. The eruption windows for these two



samples have considerable associated errors. However, the relative youth of these two lava flows is confirmed by the large amount of  $^{210}\text{Po}$  ingrowth between the first and second analyses.



TABLE 1  $^{210}\text{Po}$  data from the East Pacific Rise, 9° 50' N

| Sample   | Collection date | Location  |            | Aliquot number | Analysis Date | Total mass (g) | Aliquot mass (g) | $^{210}\text{Po}^*$ (d.p.m. g <sup>-1</sup> ) | $^{210}\text{Pb}$ (d.p.m. g <sup>-1</sup> ) | Eruption window†             |
|----------|-----------------|-----------|------------|----------------|---------------|----------------|------------------|-----------------------------------------------|---------------------------------------------|------------------------------|
|          |                 | Lat. (°N) | Long. (°W) |                |               |                |                  |                                               |                                             |                              |
| 2392-9G  | 27 May '91      | 9° 50.6'  | 104° 17.5' | 1              | 15 Aug. '91   | 8.056          | 2.094            | 0.081 ± 0.008                                 | 0.117 ± 0.006                               | 30 Dec. 1990 to 25 Feb. 1991 |
|          |                 |           |            | 2              | 3 Nov. '91    |                | 1.609            | 0.092 ± 0.010                                 |                                             |                              |
|          |                 |           |            | 3              | 27 Jan. '92   |                | 1.270            | 0.102 ± 0.013                                 |                                             |                              |
|          |                 |           |            | 4              | 31 May '92    |                | 1.268            | 0.107 ± 0.019                                 |                                             |                              |
|          |                 |           |            | 5              | 15 Jan. '93   |                | 1.772            | 0.114 ± 0.011                                 |                                             |                              |
| 2392-9WR | 27 May '91      | 9° 50.6'  | 104° 17.5' | 1              | 12 Sep. '91   | 10.238         | 3.409            | 0.107 ± 0.009                                 | 0.115 ± 0.006                               | —                            |
|          |                 |           |            | 2              | 7 Nov. '91    |                | 1.905            | 0.108 ± 0.012                                 |                                             |                              |
|          |                 |           |            | 3              | 31 May '92    |                | 0.993            | 0.111 ± 0.018                                 |                                             |                              |
|          |                 |           |            | 4              | 21 Jun. '93   |                | 1.468            | 0.115 ± 0.011                                 |                                             |                              |
|          |                 |           |            | 5              | 23 Sep. '91   |                | 1.574            | 0.082 ± 0.008                                 |                                             |                              |
| 2372-1G  | 23 Apr. '91     | 9° 50.6'  | 104° 17.5' | 2              | 18 Nov. '91   | 4.397          | 0.765            | 0.088 ± 0.015                                 | 0.006                                       | 25 Feb. to 23 Apr. 1991      |
|          |                 |           |            | 3              | 29 May '92    |                | 0.672            | 0.109 ± 0.025                                 |                                             |                              |
|          |                 |           |            | 4              | 22 Jan. '93   |                | 1.473            | 0.115 ± 0.014                                 |                                             |                              |
|          |                 |           |            | 5              | 3 Jan. '92    |                | 1.227            | 0.082 ± 0.011                                 |                                             |                              |
| 2363-7G  | 14 Apr. '91     | 9° 50.6'  | 104° 17.6' | 2              | 29 May '92    | 3.191          | 0.951            | 0.090 ± 0.012                                 | 0.005                                       | 24 Jan. to 22 Mar. 1991      |
|          |                 |           |            | 3              | 15 Feb. '93   |                | 1.008            | 0.095 ± 0.013                                 |                                             |                              |
|          |                 |           |            | 4              | 11 Jun. '92   |                | 1.240            | 0.077 ± 0.011                                 |                                             |                              |
| 2504-1G  | 12 Mar. '92     | 9° 50.3'  | 104° 17.5' | 2              | 12 Mar. '93   | 3.032          | 0.975            | 0.105 ± 0.019                                 | 0.005                                       | 2 Nov. to 29 Dec. 1991       |
|          |                 |           |            | 3              | 11 Jun. '92   |                | 1.240            | 0.077 ± 0.011                                 |                                             |                              |
| 2497-1bG | 5 Mar. '92      | 9° 53.3'  | 104° 17.9' | 1              | 11 Jun. '92   | 2.146          | 0.834            | 0.079 ± 0.012                                 | 0.145 ± 0.007                               | 7 Jan. to 5 Mar. 1992        |
|          |                 |           |            | 2              | 21 Jun. '93   |                | 1.302            | 0.135 ± 0.015                                 |                                             |                              |

Sample data for the 9° 50' N EPR lavas. 'G' refers to glass; 'WR' refers to whole rock (mesocrystalline flow interior, collected from a 3–4 cm thick band immediately below the glassy upper margin of this rock). ( $^{210}\text{Po}$ ) was measured in aliquots of a stock solution of each sample.

\* These data are corrected for procedural blanks (2–6% of the measured  $^{210}\text{Po}$  activity) and collector backgrounds (10–20 times lower than sample count rates (in d.p.m., disintegrations per min). Blanks were determined by using similar volumes of reagents and procedures as sample 2392-9G and scaled for the aliquot sizes and reagent volumes for other samples. Relatively large  $2\sigma$  counting errors result from the extremely low abundances of  $^{210}\text{Po}$ , particularly during the early stages of ingrowth. Low-activity lavas require large sample sizes, which sometimes result in coatings on the silver disks on which the sample Po is deposited for  $\alpha$ -particle counting. These coatings cause the escaping  $\alpha$ -particles to lose some of their energy before detection, resulting in broadened peaks in the  $\alpha$ -particle spectra. Under these circumstances, counting times were sometimes reduced, at the expense of lower statistical uncertainty, to avoid peak overlap between  $^{210}\text{Po}$  and the tracer,  $^{208}\text{Po}$ , which has a similar  $\alpha$ -decay energy. The extremely small concentrations (as low as  $7 \times 10^{-18}$  g  $^{210}\text{Po}$  per g rock) are too low to be detected by most other analytical methods for reasonably sized samples ( $\leq$  a few grams).

† Best-estimate eruption windows correspond to the black-filled areas in Fig. 2. Eruption window for 2392-9WR is the same as for 2392-9G.

where  $t$  is time since eruption and  $\lambda$  is the decay constant. As is customary, parentheses are used to indicate activities.

It is obvious that to calculate  $t$  from a single measurement of ( $^{210}\text{Po}$ ), one would have to know both ( $^{210}\text{Pb}$ ) and ( $^{210}\text{Po}$ ) <sub>$t=0$</sub> . For a completely degassed lava, this latter term would be zero; however, complete degassing cannot always be assumed, particularly for submarine lavas erupted under high hydrostatic pressure. But it is possible to calculate a unique maximum value for  $t$  by assuming complete degassing, and fitting an exponential function to a series of measurements made on a sample as  $^{210}\text{Po}$  grows into equilibrium with  $^{210}\text{Pb}$ . The maximum age corresponds to ( $^{210}\text{Po}$ ) = 0 on the best-fit ingrowth curve (for example, the  $y$ -intercept in a plot like those in Fig. 1).

We applied this technique to the East Pacific Rise (EPR) lavas studied here. Repeat measurements of ( $^{210}\text{Po}$ ) were made over a period of several years on aliquots of each sample and ingrowth curves with the functional form of the above equation were fitted to the data using the time interval between sequential measurements to constrain  $t$  (see Fig. 1 caption for details). The regression results yield a function for ( $^{210}\text{Po}$ ) <sub>$t$</sub> , and the known analysis times tie the results to calendar time.

If Po degassing at eruption was incomplete, the eruption time cannot be determined precisely. However, it is bracketed in all cases by the window between sample collection and the calculated maximum age. Eruption dates or 'windows' are fixed most precisely for samples with the lowest ( $^{210}\text{Po}$ ) at the time of sample collection, or for which initial ( $^{210}\text{Po}$ ) can be estimated independently, as discussed below.

All basalts analysed were collected by the submersible *Alvin* from hackly to ropey sheet flows, except sample 2497-1b which was from a lobate flow. Sample locations (Table 1) are precise to within  $\pm 20$  m. We selected for analysis three samples from

the Adventure Cruise 1 (spring 1991; samples 2363-7, 2372-1 and 2392-9) and two collected in early 1992 (2497-1 and 2504-1) because of their youthful appearance on the sea floor. Complete descriptions of the sampling locations are given elsewhere<sup>4</sup>. The three 1991 samples are from the so-called 'tubeworm barbecue' site where white 'clouds' of bacteria were emanating from numerous hydrothermal vents in April 1991. Freshly charred tubeworms and mussels were also found, some overlain by lava flows. By late May, when sample 2392-9 was taken, the area was significantly less hydrothermally active, flows had more sediment cover, bacterial activity was greatly diminished and crabs were eating the dead tubeworms. The two 1992 samples are from north and south of the 'tubeworm barbecue', which did not appear active at the time of collection. Sample 2497-1b was collected from within a new fissure to the north that was not present in 1991; sample 2504-1 was collected from just south of the 'tubeworm barbecue' site, where bacterial clouds were present in March 1992.

All samples except 2392-9 are glass fragments chipped from the upper surfaces of flows on board ship. Two (2372-1 and 2363-7) were cleaned (at sea) for 15 min in a 2M HCl ultrasonic bath. Sample 2392-9 is a whole-rock sample from which both glass and crystalline material was analysed.

Samples were analysed at Scripps Institution of Oceanography following a standard, previously reported technique<sup>12</sup>. Briefly, clean glass chips were picked using a binocular microscope and leached with 2M HCl for 15 min in an ultrasonic bath. They were then dried, weighed, spiked with a  $^{208}\text{Po}$  tracer, digested in a 1:1 mixture of concentrated HF and HNO<sub>3</sub>, dried and redissolved in 8M HCl. The single crystalline rock sample was treated similarly, except that initially it was crushed to pea-size fragments, sonified in double-distilled H<sub>2</sub>O, picked under a micro-

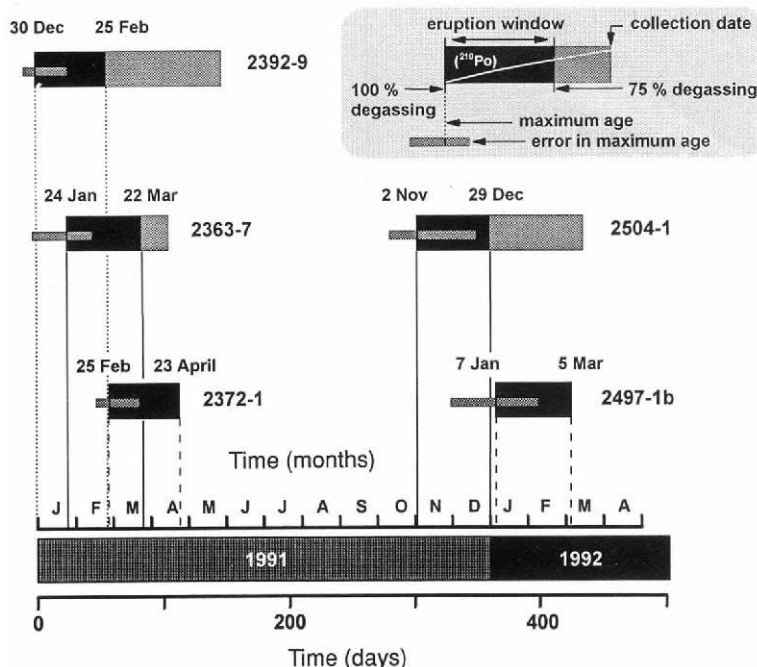


FIG. 2 Eruption windows from Fig. 1 plotted against calendar time. A legend is given in the inset. The black bars denote our best estimate for the actual eruption windows and are bounded by the dates of maximum (100%) and minimum (75%) extents of initial Po degassing (as discussed in the text). The corresponding part of a  $^{210}\text{Po}$  ingrowth curve is also given in the inset. It is obvious that there were two eruptive episodes separated by  $\sim 7$  months along this section of the EPR.

scope, and powdered in a tungsten carbide ball mill before dissolution.

Sample solutions were stored in clean, acid-leached high-density polyethylene bottles before analysis.  $^{210}\text{Po}$  activities were measured periodically in aliquots of these solutions over  $\sim 2$  yr following dissolution. The analytical results are summarized in Table 1 and the  $^{210}\text{Po}$  ingrowth curves are shown in Fig. 1. All data are corrected for collector backgrounds and procedural blanks, as discussed in Table 1 caption. The grey regions in Fig. 1 (at the left of each plot) are the maximum 'eruption windows' discussed earlier; they are plotted relative to calendar time in Fig. 2.

Because all these mid-ocean ridge basalts (MORBs) have very similar chemical compositions and were erupted at the same water depth, both factors which should govern degassing efficiency, it is reasonable to expect that they all had very similar initial ( $^{210}\text{Po}$ ). This should be true in general for any normal MORB sample set collected from a restricted depth range. However, the degassing extent may or may not be as high as the subaerial value of 100%. Two of the samples analysed here (2372-1 and 2497-1b) had  $^{210}\text{Po}$  contents at the time of their collection that were only  $\sim 25\%$  of the secular equilibrium value (25.1% and 25.6%, respectively), suggesting that all samples were at least 75% degassed during eruption. This condition constrains the eruption window to lie within that part of the ingrowth curve between 0 and 25% of the  $^{210}\text{Pb}$  activity. Eruption windows using this criterion are given in Table 1 and Fig. 2.

These best-estimate eruption windows are  $\sim 2$  months long, and allow investigation of MORB eruption chronology on a much finer scale than was possible heretofore. If Po degassing is typically considerably larger than our (conservative) minimum estimate of 75%, the resolution of this technique may improve further. Such improvement will require analyses of  $^{210}\text{Po}$  in several very young MORBs with known eruption dates in order to determine the normal degree of degassing. Remote observations of potential eruption indicators that fall within an eruption window (for example, seismicity<sup>18</sup> or megaplumes<sup>2,19</sup>) might also be used further to constrain eruption dates and the range of initial ( $^{210}\text{Po}$ ) in MORBs.

Eruption windows for samples 2504-1 and 2497-1b are less certain than those for other samples because we were unable to make more than two  $^{210}\text{Po}$  analyses on each of these lavas (see Fig. 1 caption). Nevertheless, the results clearly show that they

are younger than the eruptions of early 1991 and must have erupted between the 1991 and 1992 expeditions. Thus our  $^{210}\text{Po}$ – $^{210}\text{Pb}$  dating studies show unequivocally that a small section of the EPR experienced eruptions—probably several—during the first few months of 1991, and again during late 1991/early 1992. This type of information is crucial for assessing the frequency and volume of eruptions within an eruptive cycle, the short-term variation in MORB chemistry within a small area, whether changes in lava chemistry and hydrothermal vent fluid chemistry are related, and how quickly biological communities respond to the changes caused by eruption. At a minimum, our data suggest that at least two pulses of eruptive activity occurred, roughly a year apart, at a single location on the ridge. MORB eruptions at this locality are clearly not single events separated by hundreds or thousands of years of quiescence.

All three lavas with  $^{210}\text{Po}$  eruption windows in the first few months of 1991 are mafic ( $>8.5$  wt% MgO) MORB and have nearly identical chemical compositions. The Po data would be consistent with their being separate samples of a single flow only in the unlikely event that the degree of degassing was extremely variable at the time of eruption, from complete for 2372-1 to 75% for 2392-9G. Also, although they were all collected close to each other, at least one (2363-7) was collected from a physically different flow unit. The data suggest that the samples are from several different flows, erupted from a homogeneous magma reservoir over a period of  $\sim 4$  months, as part of a single episode.

In contrast to those from early 1991, the two younger lavas are chemically distinguishable. Their eruption windows are also distinct, indicating that the flows were separate events, derived from a different magma reservoir or with an intervening change in lava composition. Sample 2504-1 was collected from the same area as the 1991 flows and is nearly identical to them chemically. This implies that the magma reservoir supplying lavas to the 'tubeworm barbecue' site maintained essentially constant composition over at least 1 yr. Sample 2497-1b was collected  $\sim 5$  km to the north; it is therefore unclear whether its different chemical composition is a temporal or spatial phenomenon. The younger age of this flow is consistent, however, with other observations that suggest that volcanic activity has shifted northward into a region that had not recently been active<sup>5,20,21</sup>.

The observation that Po degases from erupting mid-ocean ridge lavas implies that similar mechanisms may also operate



for other volatile and moderately volatile metals. If this is the case, estimates of their fluxes to seawater based only on data from mid-ocean ridge hydrothermal systems would be lower limits. But the degassing process may affect only the surfaces of flows, because Po in the single mesocrystalline sample we analysed was <20% degassed on eruption. Future studies should evaluate the fine-scale variations in Po and other volatiles, such as CO<sub>2</sub> and volatile metals, with depth in submarine flows.

The importance of the <sup>210</sup>Po-<sup>210</sup>Pb dating discussed here is that it not only confirmed the suspicions<sup>4</sup> of observers in the submersible *Alvin* that mid-ocean ridge eruptions had taken place shortly before their April 1991 visit to 9° 50' N on the EPR, but that it provided the only information about the number and

timing of eruptions as well. When the same area was revisited after a 9-month hiatus, much of the physical evidence for recent volcanic activity had disappeared, although other nearby regions appeared to be active<sup>5</sup>. Along such portions of the mid-ocean ridge system, the <sup>210</sup>Po-<sup>210</sup>Pb chronometer has the potential to provide information about the temporal variability of submarine eruptions with unprecedented time resolution, particularly when used together with detailed direct and remote observations. This chronometer, when combined with other recently developed radioactive tracers of MORB eruptions (for example, refs 22–24), will undoubtedly allow for even greater understanding of the temporal variability of submarine eruptions and their effect on the geology and biology of the ridge ecosystem. □

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## New whale from the Eocene of Pakistan and the origin of cetacean swimming

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**MODERN whales (order Cetacea) are marine mammals that evolved from a land-mammal ancestor, probably a cursorial Palaeocene–Eocene mesonychia<sup>1–3</sup>. Living whales are streamlined, lack external hind limbs, and all swim by dorsoventral oscillation of a heavily muscled tail<sup>4,5</sup>. A streamlined rigid body minimizes resistance, while thrust is provided by a lunate horizontal fluke attached to the tail at a narrow base or pedicle<sup>6</sup>. We describe here a new 46–47-million-year-old archaeocete intermediate between land mammals and later whales. It has short cervical vertebrae, a reduced femur, and the flexible sacrum, robust tail and high neural spines on lumbar and caudals required for dorsoventral oscillation of a heavily muscled tail. This is the oldest fossil whale described from deep-neritic shelf deposits, and it shows that tail swimming evolved early in the history of cetaceans.**

The early evolution of whales is illustrated by partial skulls and skeletons of five archaeocetes of Ypresian (early Eocene) and Lutetian (early Middle Eocene) age (Table 1), all found in sediments deposited near or in the ancient Tethys Sea. *Pakicetus*<sup>7–10</sup> is the oldest fossil whale known, and it is also the smallest early archaeocete, with a skull and auditory bullae about half the size of those of the other four genera. All have dense auditory bullae, showing that they were at least partially aquatic, but *Pakicetus* lacks anatomical features required for directional and efficient hearing in water<sup>8,10</sup>. *Ambulocetus*<sup>11</sup>, the

earliest marine archaeocete known to date, has a long femur and long foot and is interpreted to have been an otter- or seal-like foot-propelled swimmer<sup>11</sup>. *Indocetus*<sup>12</sup> resembles *Ambulocetus* in having long hind limbs, but it also has a robust sacrum with four solidly fused centra and large proximal tail vertebrae, suggesting that it was a caudally propelled swimmer<sup>13</sup>. *Protocetus*<sup>14–16</sup> is more advanced in having a reduced single-centrum sacrum, meaning that its lumbocaudal trunk was flexible like that of modern whales. *Protocetus* has a sacrum that did not articulate directly with the pelvis<sup>14</sup>, and it must have been fully aquatic because it could not support its weight on land. *Rodhocetus* described here is the first early archaeocete with a complete thoracic, lumbar and sacral vertebral column. It retains primitive features seen in land mammals, but also exhibits derived characteristics found only in later cetaceans.

Order Cetacea  
Suborder Archaeoceti  
Family Protocetidae

*Rodhocetus kasrani*, new genus and species

**Etymology.** *Rodho*, geological anticline where the type was found, and *cetus*, Latin, whale (masc.); *kasrani*, Baluch tribe inhabiting the type area.

**Holotype.** Geological Survey of Pakistan/University of Michigan [GSP-UM] 3012, cranium and lower jaws found with a partially articulated postcranial skeleton including cervical vertebrae C2–C7, thoracics T1–T13, lumbar L1–L6, sacra S1–S4, caudals Ca1–Ca4, most ribs, sternal manubrium and xiphisternum, left and right os coxae, and left femur (Fig. 1). Tooth wear and fusion of vertebral epiphyses show specimen to be fully adult. Type found by X. Zhou in 1992 is conserved at the Geological Survey of Pakistan, Islamabad.

**Referred specimen.** GSP-UM 1852, left and right dentaries with much of the lower dentition, collected in 1981 by W. Ryan 1.6 km north of Rakhi Nala and 3 km west of Rakhi Munh rest house, 90 km southwest of the type locality (29°58'06" N, 70°06'43" E, 39 K/1 15' quadrangle).

**Type locality.** Middle of valley drained by Bozmar Nadi, Rodho anticline of Zinda Pir anticlinorium, Sulaiman Range, near the

TABLE 1 Skull and femur measurements of *Rodhocetus kasrani* compared with those of other early archaeocetes

|                                          | <i>Pakicetus inachus</i> <sup>7-10</sup><br>Lower Kuldana Fm.<br>Pakistan<br>Late Ypresian<br>Riverine<br>TA3.1 low-stand*<br>~49.5–49.0 Myr*<br>(~52.5–52.0 Myr)† | <i>Ambulocetus natans</i> <sup>11</sup><br>Upper Kuldana Fm.<br>Pakistan<br>Ypresian–Lutetian<br>Shallow marine<br>TA3.1–TA3.2<br>~49.0–48.5 Myr<br>(~52.0–51.5 Myr) | <i>Rodhocetus kasrani</i><br>Lower Domanda Fm.<br>Pakistan<br>Early Lutetian<br>Deep neritic<br>TA3.2–TA3.3<br>~46.5 Myr<br>(~49.5 Myr) | <i>Indocetus ramani</i> <sup>12,13</sup><br>Upper Domanda Fm.<br>Pakistan<br>Early Lutetian<br>Shallow marine<br>TA3.3 shelf-margin<br>~46.5–46.0 Myr<br>(~49.5–49.0 Myr) | <i>Protocetus atavus</i> <sup>14-16</sup><br>Lower Mokattam Fm.<br>Egypt<br>Middle Lutetian<br>Deep neritic<br>TA3.3 high-stand<br>~46.0–45.0 Myr<br>(~49.0–48.0 Myr) |
|------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cranium                                  |                                                                                                                                                                    |                                                                                                                                                                      |                                                                                                                                         |                                                                                                                                                                           |                                                                                                                                                                       |
| Condylobasal length (cm)                 | 30.0–32.0‡                                                                                                                                                         | —                                                                                                                                                                    | 61.5                                                                                                                                    | 62.0‡                                                                                                                                                                     | 58.0                                                                                                                                                                  |
| Width across supraorbital processes (cm) | —                                                                                                                                                                  | —                                                                                                                                                                    | 19.5                                                                                                                                    | 20.0–21.0                                                                                                                                                                 | 16.7                                                                                                                                                                  |
| Width across zygomatic arches (cm)       | 14.5                                                                                                                                                               | —                                                                                                                                                                    | 26.0                                                                                                                                    | 26.0‡                                                                                                                                                                     | 24.0                                                                                                                                                                  |
| Width across exoccipitals (cm)           | 9.1                                                                                                                                                                | —                                                                                                                                                                    | 22.0                                                                                                                                    | 22.0                                                                                                                                                                      | —                                                                                                                                                                     |
| Auditory bulla length (mm)               | 25.5                                                                                                                                                               | 63.0‡                                                                                                                                                                | 60.0                                                                                                                                    | 62.0–70.0                                                                                                                                                                 | 54.0–55.0                                                                                                                                                             |
| Auditory bulla width (mm)                | 22.5                                                                                                                                                               | 45.0                                                                                                                                                                 | 46.0                                                                                                                                    | 45.0                                                                                                                                                                      | 40.0                                                                                                                                                                  |
| M <sup>1</sup> crown length (mm)         | 17.3                                                                                                                                                               | 25.0                                                                                                                                                                 | 24.3                                                                                                                                    | 21.5‡                                                                                                                                                                     | 22.0                                                                                                                                                                  |
| Mandible                                 |                                                                                                                                                                    |                                                                                                                                                                      |                                                                                                                                         |                                                                                                                                                                           |                                                                                                                                                                       |
| Mandibular fossa height (mm)             | Small                                                                                                                                                              | —                                                                                                                                                                    | 65.0 (large)                                                                                                                            | —                                                                                                                                                                         | —                                                                                                                                                                     |
| Mandibular symphysis length (cm)         | —                                                                                                                                                                  | —                                                                                                                                                                    | 22.0§                                                                                                                                   | —                                                                                                                                                                         | —                                                                                                                                                                     |
| M <sub>3</sub> crown length (mm)         | 17.8                                                                                                                                                               | —                                                                                                                                                                    | 24.5                                                                                                                                    | —                                                                                                                                                                         | —                                                                                                                                                                     |
| M <sub>3</sub> crown width (mm)          | 8.9                                                                                                                                                                | —                                                                                                                                                                    | 13.8                                                                                                                                    | —                                                                                                                                                                         | —                                                                                                                                                                     |
| M <sub>3</sub> crown height (mm)         | 12.0                                                                                                                                                               | —                                                                                                                                                                    | 28.7                                                                                                                                    | —                                                                                                                                                                         | —                                                                                                                                                                     |
| Femur                                    |                                                                                                                                                                    |                                                                                                                                                                      |                                                                                                                                         |                                                                                                                                                                           |                                                                                                                                                                       |
| Head diameter (acetabulum; cm)           | —                                                                                                                                                                  | —                                                                                                                                                                    | 3.1 (3.2)                                                                                                                               | (4.0)                                                                                                                                                                     | —                                                                                                                                                                     |
| Total length (cm)                        | —                                                                                                                                                                  | 28.0                                                                                                                                                                 | 17.8                                                                                                                                    | 25.0‡                                                                                                                                                                     | —                                                                                                                                                                     |

\* Correlations and ages in million years based on planktonic foraminiferans<sup>18,19</sup>, nannofossils<sup>20</sup> and sea-level sequence stratigraphy with radiometric calibration of Haq et al.<sup>17</sup>. † Alternative ages in parentheses reflect timescale of Berggren et al.<sup>23</sup>. ‡ Estimate. § Mandibular symphysis extends to point just posterior to P<sub>2</sub>.

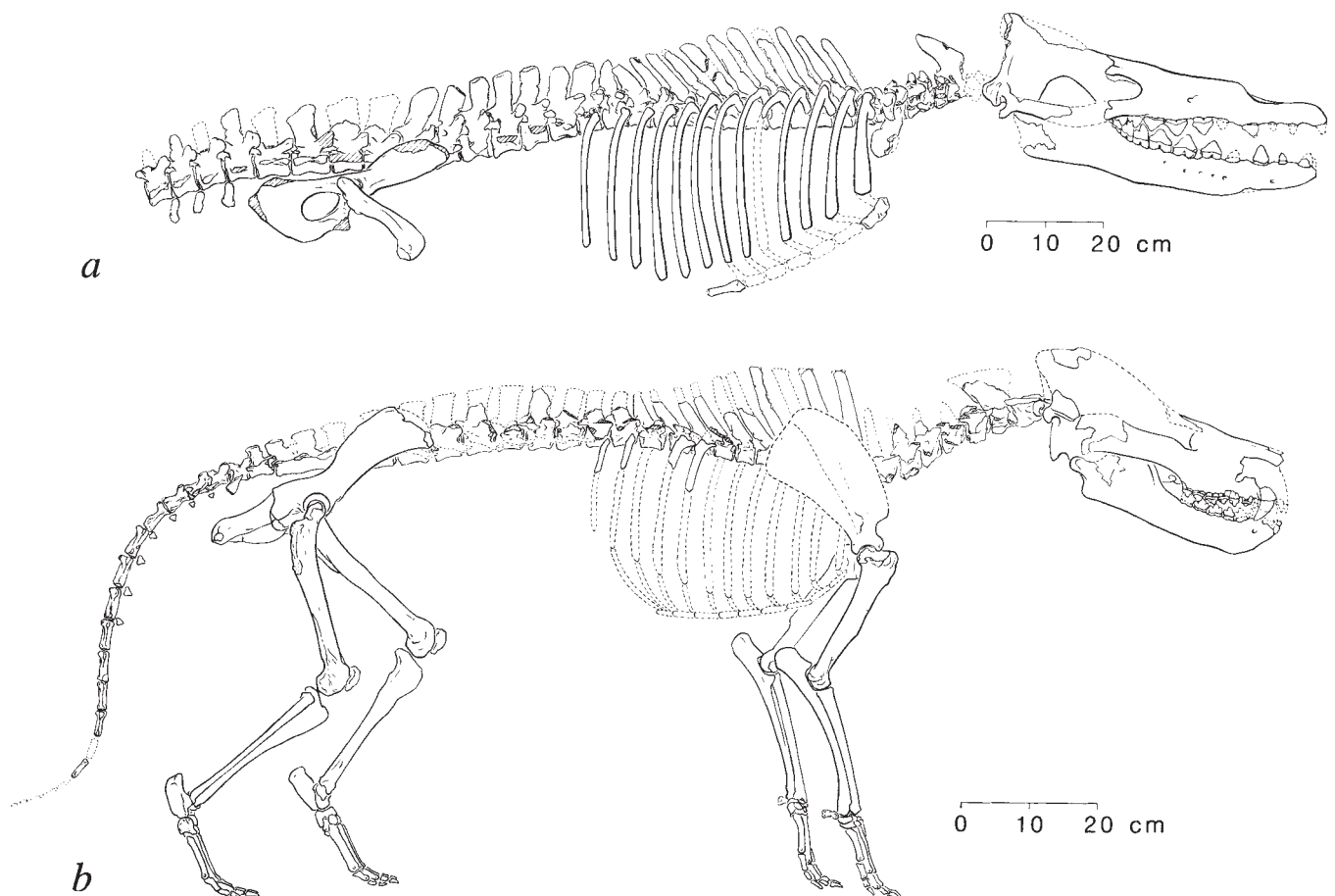
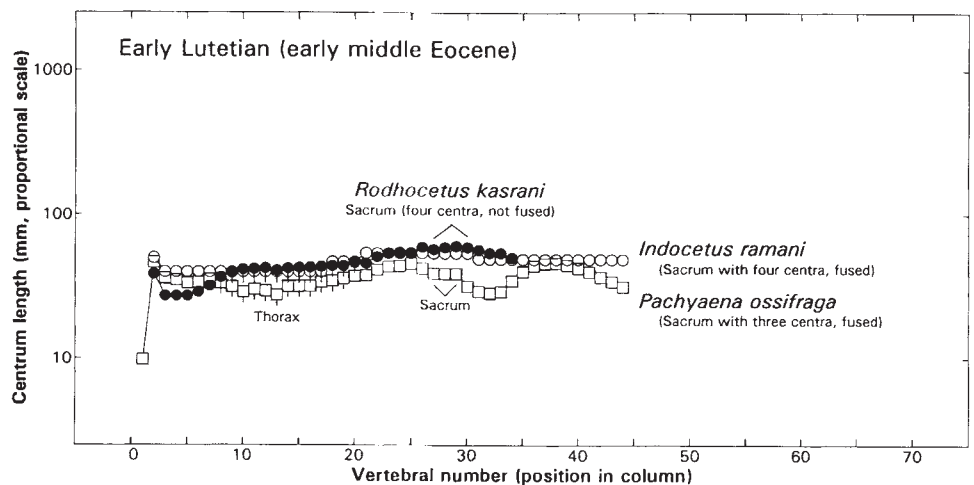


FIG. 1 Skeleton of early Middle Eocene cetacean *Rodhocetus kasrani* (a, holotype GSP-UM 3012) compared with early Eocene mesonychid *Pachyaena ossifraga* (b, ref. 3) representing the land-mammal group thought to be ancestral to whales<sup>1,2</sup>. Both are drawn to the same thorax + lumbus length in right lateral view (some elements are reversed from left side). Dashed lines and cross-hatching show reconstructed parts. *Rodhocetus* forelimbs, distal hind limbs and distal tail are not yet known (these were removed from the type before burial, possibly

by scavenging sharks). The skeleton of *Rodhocetus* is 2 m long, as preserved, and in life, with a full tail, it may have been as long as 3 m. Note large typically protocetid skull, short neck, vertebral count of 7 cervicals, 13 thoracics, 6 lumbar, 4 sacral, and 4+ caudals and long chevrons ventral to caudals. Thoracics T11 and T12 have overlapping neural spines, T12 is anticlinal, and T13 is diaphragmatic. The thorax anterior to T13 was relatively rigid, whereas the skeleton posterior to T13 was more flexible (enhanced by loss of fusion of sacral vertebrae).



FIG. 2 Length-of-vertebrae profile of *Rodhocetus kasrani* (solid circles) superimposed on that of land mammal *Pachyaena ossifraga* (squares; ref. 3) and archaeocete *Indocetus ramani* (empty circles; ref. 13). *Rodhocetus* profile is typically cetacean<sup>22</sup> in having short cervical vertebrae (here vertebral numbers 2–7, note difference from *Indocetus*) and long posterior lumbar, sacral and anterior caudals (here vertebral numbers 25–34, note difference from *Pachyaena*).



southwestern corner of Punjab Province, Pakistan (30°46'06" N, 70°26'37" E, 39 J/5 Dhodak 15' quadrangle).

**Age and distribution.** Both specimens are from green shales in the lower part of the Domanda Formation deposited in the transition from a high-stand to a shelf-margin sediment wedge interpreted as the transition from Tejas sea-level cycle TA3.2 to TA3.3 (ref. 17) on the basis of planktonic foraminiferans<sup>18,19</sup> and nannofossils<sup>20</sup>. The top of TA3.2 and base of TA3.3 are early Lutetian in age, early Middle Eocene, and the interval yielding *Rodhocetus* is calibrated at about 46.5 Myr<sup>17</sup>. Both specimens come from marine sediments in the Sulaiman Range of Pakistan but *R. kasrani* was probably widely distributed along the southern margin of ancient Tethys.

**Diagnosis.** *Rodhocetus* differs from *Pakicetus* and other Ypresian archaeocete species in having higher-crowned cheek teeth, larger auditory bullae, and larger mandibular foramina and mandibular canals. It differs from *Ambulocetus* in having higher neural spines on lumbar vertebrae and a much shorter femur, and it differs from *Indocetus ramani* in having a convex posterior surface of the exoccipital, shorter cervical vertebrae, unfused sacral vertebrae and a shorter femur. *Rodhocetus* differs from *Protocetus atavus* and all later whales in having external nares open above the canines and in retaining four vertebrae in the sacrum, with a larger pelvis joined to the sacrum by a normal land-mammal synarthrosis.

The skull of *Rodhocetus* is large relative to the rest of the skeleton (Fig. 1), and it has the elongated premaxillae and dentaries, broad frontal shield, high nuchal crest, and other proportions typical of archaeocete cetaceans<sup>15</sup> (comparative measurements in Table 1). External nares open above the upper canine. Infraorbital foramina are small. The posterior surface of the exoccipital is convex like that of *Protocetus*. Auditory bullae are large and dense, but there are no pterygoid fossae or accessory air sinuses associated with these. There is a well-developed mandibular foramen or fossa on the medial side of each dentary, with an 'acoustic window' measuring about 90 mm long by 65 mm high (bone lateral to this is 4.0 mm thick) interpreted as a wave guide to aid hearing in water<sup>21</sup>. Teeth are robust; lower molars have crowns higher than they are long (Table 1).

GSP-UM 3012 is the only early archaeocete showing the number of cervical (7), thoracic (13), lumbar (6) and sacral (4) vertebrae in the skeleton. Four caudals are preserved, but the total number is unknown. Cervical centra are shorter than those of anterior thoracics; C3–C5 each measure about 27 mm in centrum length. Anterior thoracics average about 42 mm in centrum length, posterior thoracics average 45 mm, lumbar average 54 mm, sacral average 59 mm, and proximal caudals average 54 mm. The vertebral length profile for *R. kasrani* is compared

with profiles for the land mammal *Pachyaena ossifraga*<sup>3</sup> and archaeocete *I. ramani*<sup>13</sup> in Fig. 2, which shows *R. kasrani* to have had shorter cervicals and longer sacra than expected from either comparison. *Rodhocetus* differs from *Pachyaena* and *Indocetus* in having unfused sacral vertebrae (Fig. 3).

No forelimb elements of *Rodhocetus* have been found to date. High neural spines on anterior thoracics and long ribs give *Rodhocetus* a deep narrow thorax (measuring about 45 × 35 cm at T9). High neural spines on anterior thoracics are found in mammals such as *Pachyaena* that cantilever a heavy head and thorax above ground with the forelimbs acting as piers.

The pelvis is well preserved in GSP-UM 3012. Os coxae measure 336 mm in length and 128 mm dorsoventrally, and the

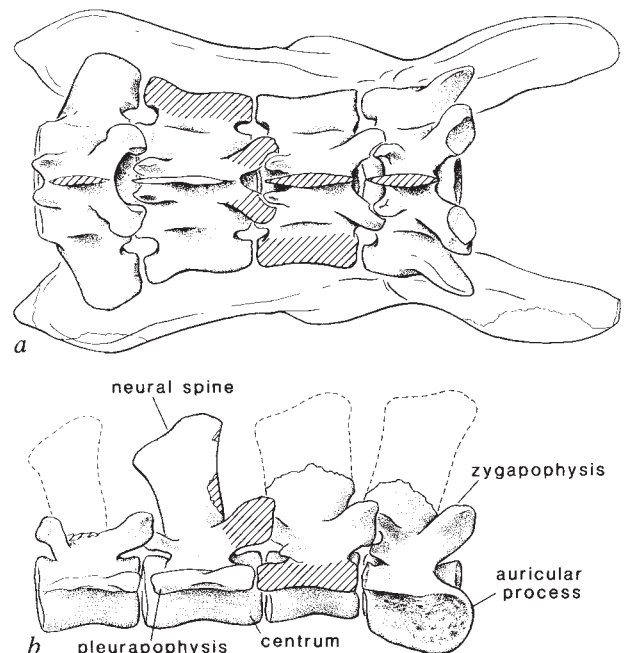


FIG. 3 Sacrum of *Rodhocetus kasrani*, GSP-UM 3012 (holotype), in a, dorsal view, superimposed on outline of pelvis, and b, right lateral view. Note well-developed pleurapophyses surrounding sacral foramina, but asynostosis of centra, neural spines, zygapophyses and pleurapophyses that are usually solidly fused in a mammalian sacrum. Tooth wear and fusion of vertebral epiphyses show specimen to be adult, so absence of fusion is not due to an early stage of ontogenetic development.

large central acetabulum is 13 mm deep. The ilium is conspicuously short compared with that of *Pachyaena*, indicating less powerful hip extension. The obturator foramen is large, measuring 67 mm in greatest diameter and 47 mm in least diameter. Pubes join at a short narrow symphysis. The pelvis of *Rodhocetus* articulates with the vertebral column by normal mammalian sacral synarthroses, meaning that *Rodhocetus* could support its body weight on land.

The femur of *R. kasrani* is only 60–70% as long as that in the similar-sized land mammal *P. ossifraga*<sup>3</sup> and early archaeocetes *Ambulocetus natans* and *I. ramani* (Table 1). The femoral head is large and spherical, with a deep fovea capitis femoris for the round ligament (lost in later whales<sup>22</sup>). The greater trochanter is slightly higher than the head. The lesser trochanter is large and medially directed. The intertrochanteric crest is well developed and encloses a deep trochanteric fossa. There is no third trochanter. The patellar groove is well developed. The lateral condyle is larger than the medial condyle, but both are large and project substantially from the body of the femur. Both condyles are directed posteriorly. Nothing is known of the rest of the hind limb.

*Rodhocetus* is important for three reasons. First, it is an early archaeocete that retains high neural spines on anterior thoracics and a pelvis articulating directly with the sacrum. These are primitive characteristics of mammals that support their weight on land, and both suggest that *Rodhocetus* or an immediate predecessor was still partly terrestrial. At the same time, cervicals are short, enhancing rigidity of the anterior body, sacra are large but unfused, enhancing power and flexibility, and the femur is reduced, streamlining the lumbocaudal trunk. These are derived characteristics of later archaeocetes and modern whales associated with aquatic locomotion. Thus the morphology of *Rodhocetus* is intermediate, as might be expected of a transitional form evolving from land to sea.

Second, *Rodhocetus* is the oldest fossil whale described from deep-sea shelf deposits. Contemporary and slightly younger whales such as *Indocetus*, which inhabited shallower water, retained long hind limbs and fused sacral vertebrae, indicating that there was significant morphological and locomotor diversity in early archaeocete evolution. Fossils found in shallow marine environments may not represent the full range of this diversity.

Finally, most modern whales have robust lumbar and proximal caudal centra, and all lack fusion of sacral vertebrae, making the lumbocaudal column seamlessly flexible. High dorsal neural spines and long ventral chevrons limit vertebral excursion but provide leverage for powerful axial and abdominal muscles<sup>4–6</sup>. *Rodhocetus* has all of these functional features. This indicates that the characteristic cetacean mode of swimming by dorsoventral oscillation of a heavily muscled tail evolved within the first three million years or so of the appearance of archaeocetes. Terminal caudals are lacking in the type specimen of *Rodhocetus* and we cannot assess the possible presence of a caudal fluke, but it is reasonable to expect development of a fluke to coincide with shortening of the neck, flexibility of the sacrum and reduction of hind limbs first observed in *Rodhocetus*. This idea can be tested when a more complete tail of *Rodhocetus* is found. □

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## Interocular control of neuronal responsiveness in cat visual cortex

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NEURONS in the cat primary visual cortex are selective for particular contour orientations<sup>1</sup> but their responsiveness can vary under certain conditions. After prolonged stimulation (adaptation), the contrast sensitivity of cortical cells is reduced<sup>2–5</sup> and the 'gain' (the strength of response as a function of contrast) falls<sup>5,6</sup>. The response to an optimal contour is also reduced when a different stimulus is superimposed on the receptive field in the same eye<sup>7–9</sup>. Here we report that the sudden appearance of an inappropriate stimulus in one eye can interocularly suppress the activity of cortical neurons if they are already responding to an optimally oriented stimulus in the other eye. In strabismic cats, whose cortical neurons lack binocular facilitation, even contours of similar orientation shown to the two eyes trigger such suppression. This interocular control of cortical responsiveness could serve to veto signals from one eye under conditions that would otherwise cause double vision and perceptual confusion.

If both eyes are stimulated with contours of similar orientation, most neurons in the visual cortex of normal cats exhibit binocular summation or facilitation, as long as the relative location of the images on the two retinæ (retinal disparity) is optimized: such interocular interactions are thought to play a part in binocular fusion and stereoscopic vision<sup>10–12</sup>. When human observers view conflicting stimuli in the two eyes they usually experience alternating perceptual suppression (binocular rivalry). However, for cortical neurons the response to an optimal stimulus in one eye is hardly affected when a stimulus of orthogonal orientation is presented simultaneously in the other eye<sup>9,13</sup>.

We studied the orientation-dependence of binocular interaction in cat striate cortex with a new procedure in which a series of grating stimuli of different orientations were presented to one eye while the recorded neuron was continuously responding to an optimally oriented drifting grating presented to the other eye. For 42 of the 45 binocular neurons studied in five normal cats (see legend to Fig. 1), responses to the continuous 'conditioning' stimulus were significantly augmented, as expected, when the other eye was shown a stimulus matched in orientation to the optimal, as long as the spatial phase relationship between the two was appropriate (Fig. 1a), and such binocular facilitation decreased with increasing difference in orientation between the two eyes<sup>14</sup>. However, for most neurons (25 out of 45), the sudden introduction of a stimulus of orthogonal orientation significantly depressed the response to the optimal conditioning stimulus (Fig. 1a). We wondered whether the

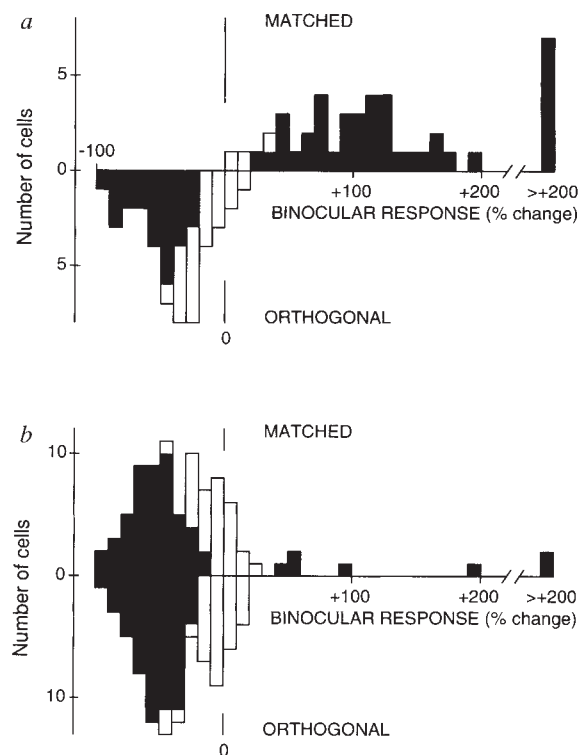
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FIG. 1 These histograms show the types of binocular interaction observed in the visual cortex of normal cats (a) and strabismic animals (b) when stimulated with gratings of either the same orientation ('matched') or orthogonal orientations in the two eyes. For these results, the dominant eye was stimulated continuously with a 'conditioning' grating of optimal orientation, spatial frequency and temporal frequency of drift, while gratings of the same spatial and temporal frequency but varying in orientation were presented intermittently to the other eye, in a pseudorandom series. The periods of binocular stimulation lasted 5 s, each preceded by a 5-s period of dominant eye stimulation alone. The abscissa plots the magnitude of response (impulses  $s^{-1}$ ) during binocular stimulation (mean of at least 4 presentations) as a percentage of the mean response during the immediately preceding periods of monocular stimulation: positive values indicate enhancement of the response, negative values suppression. The ordinate is the number of cells: data obtained with matched stimuli are plotted upwards and those for orthogonal gratings plotted downwards. Filled blocks indicate cells whose binocular responses deviated significantly from the control monocular level, indicated by zero on the abscissa. For the 45 cells from normal cats, only three did not exhibit significant enhancement for matched gratings (optimized in relative spatial phase) whereas most were clearly suppressed by crossed orientations (independent of relative spatial phase). Only 7 out of the 85 cells studied in strabismic animals displayed significant binocular enhancement for matched gratings and most showed clear suppression for both matched and orthogonal stimuli. Only one of the strabismic cats (one of the exotropes) appeared to fixate alternately with the two eyes, when tested before the recording experiment, but all data have been pooled because there was no obvious difference between the results from this animal and the others.

**METHODS.** Standard electrophysiological techniques for single-cell recording were used<sup>30</sup>. For surgery, cats were anaesthetized with intravenous alphaxalone/alphadolone: during recording they were anaesthetized and paralysed with a continuous infusion of sodium pentobarbitone ( $1-2 \text{ mg kg}^{-1} \text{ h}^{-1}$ ) and gallamine triethiodide ( $10 \text{ mg kg}^{-1} \text{ h}^{-1}$ ). EEG and ECG were constantly recorded to assess the state of anaesthesia. Room air was used for artificial ventilation, with added carbon dioxide to bring end-expiratory  $\text{CO}_2$  to 4.5–5%. The eyes were protected with contact lenses and optimally refracted. Visual stimuli were presented on two cathode-ray tube displays, viewed dichoptically with front-silvered mirrors, which were adjusted in position



to bring the receptive fields of isolated units in either eye to the centre of the two screens. Drifting, sinusoidally modulated gratings (mean luminance  $17.5 \text{ cd m}^{-2}$ ) were generated by a 'Picasso' (Innisfree) image synthesizer. The 'Picasso' as well as data acquisition and analysis were externally controlled by a 'Visual Stimulation' software package (Cambridge Electronic Design). Tuning curves for orientation and spatial frequency were determined monocularly through either eye, before binocular interactions were tested.

particular temporal sequence of stimulation used in our procedure might be responsible for the suppression that we saw, and what form of gain change is involved.

To investigate these questions, we measured responses during brief (5 s) periods of binocular stimulation in which an optimal drifting grating was presented to the dominant eye together with an orthogonal grating shown to the other eye. The contrast of the dominant eye's grating was varied from presentation to presentation in a randomized sequence to generate contrast-response functions (Fig. 2), while the contrast of the orthogonal grating was held constant. During the 5-s periods preceding binocular exposures there was either (1) a blank screen shown to both eyes; (2) the orthogonal grating alone in the non-dominant eye; or (3) the optimal grating alone in the dominant eye. During the periods of measurement, stimulation was identical under all three conditions. But only in the last condition, in which the conflicting stimulus was introduced against a pre-existing response to the optimal stimulus (filled circles), was the activity of the cell consistently depressed below the corresponding control (unfilled circles; see legend to Fig. 2).

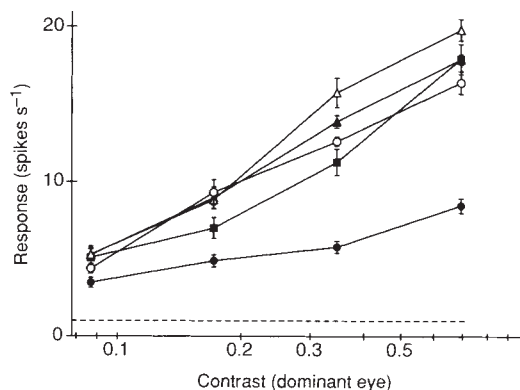
This phenomenon is predominantly manifested as a change in the slope of the contrast gain function; that is, response strength is reduced by roughly the same percentage at all supra-threshold contrasts used. Although the threshold contrast (the value of contrast for zero response) was not specifically determined, it appears from extrapolation of the contrast-response curves to the level of background activity that threshold is not substantially increased during suppression, unlike in the gain changes resulting from adaptation or conflicting monocular stimulation, which are characterized by horizontal shifts of the contrast-response function along the contrast axis without an obvious change in slope<sup>6,15</sup>.

Interocular suppression of this sort might be involved in binocular rivalry in humans. A stimulus suddenly introduced into one eye while the other eye is already viewing a different pattern does indeed lead to the perceptual suppression of the latter and 'capture' of perception by the newly presented stimulus. In normal humans, interocular suppressive interactions are evident only when the two eyes are stimulated with conflicting patterns. Such situations occur in normal viewing of three-dimensional scenes containing a large range of binocular disparities. For parts of the scene much closer and further than the fixation point, corresponding retinal areas receive the images of different patterns, but double vision and confusion are rarely noticed, implying that one of the images can be suppressed.

Now, for humans with strabismus (squint), in which the visual axes point in different directions, stereoscopic vision is compromised, and many such patients experience powerful interocular suppression independent of the relative orientation of contours in the two eyes<sup>16</sup>. In the primary visual cortex of cats made artificially strabismic early in life, neurons generally lack excitatory binocular interactions<sup>17,18</sup>, which presumably accounts for the failure of stereopsis. We recorded from 85 striate cortex neurons in five strabismic cats (three convergent, two divergent) and found that, for 47 cells, responses to an optimal stimulus already present in the dominant eye were significantly reduced by introducing a grating to the non-dominant eye, regardless of its orientation (Fig. 1b). This orientation-independent interocular suppression was seen in animals with convergent as well as with divergent squint. It exhibits the same gross non-linearity as does the suppression for orthogonally oriented gratings seen in normal animals in that it depends on the immediate history of visual stimulation.

Figure 3 shows contrast-response functions, determined as

FIG. 2 Contrast-response functions for a simple cell recorded in layer 2/3 of a normal cat. For each function, the contrast of a drifting grating of optimal orientation and spatial frequency shown to the dominant eye was randomly varied in an interleaved series. For all curves, data from 6 trials were averaged (mean  $\pm$  s.e.m.). For one control curve (unfilled triangles) the dominant eye alone was stimulated for 5 s, with the other eye viewing a blank screen of the same mean luminance, preceded by a blank screen to both eyes. For another control (unfilled circles), the dominant eye was stimulated for 10-s periods and the response was measured during the second 5 s of each presentation, in order to control for adaptation during prolonged stimulation. The small difference between the two control curves for contrast values higher than 0.2 is presumably due to such adaptation. The three other curves all plot responses during 5-s periods of binocular stimulation in which an orthogonally oriented drifting grating (contrast = 0.7) was presented to the non-dominant eye, together with the optimum grating (of varied contrast) shown to the dominant eye. The only difference between the three conditions was in the nature of stimulation during the 5-s period immediately preceding each measurement, which was as follows. Filled circles: optimal grating presented alone to the dominant eye, at the same contrast as during the binocular exposure; filled squares: orthogonal grating presented alone to the other eye; filled triangles: blank screen presented to both eyes, with simultaneous onset at the start of the period of binocular stimulation. The interrupted line indicates the mean level of spontaneous discharge during presentation of blank



for Fig. 2, for a representative monocularly driven neuron from a cat with convergent squint. In this case, the gratings presented to the two eyes were of the same orientation, optimal for the dominant eye. Once again, response strength was clearly depressed only when the potentially suppressive stimulus was introduced while the cell was already responding through the dominant eye. However, unlike in normal animals, even an identical pattern presented to the silent eye suppressed the response to a pre-existing stimulus in the dominant eye, rather than eliciting facilitation, whatever the relative spatial phase. Perhaps interocular suppression is independent of relative orientation even in normal animals, but is swamped by powerful facilitatory interactions for matched stimuli.

Alternations of such interocular suppression, perhaps triggered by changes in retinal stimulation resulting from eye move-

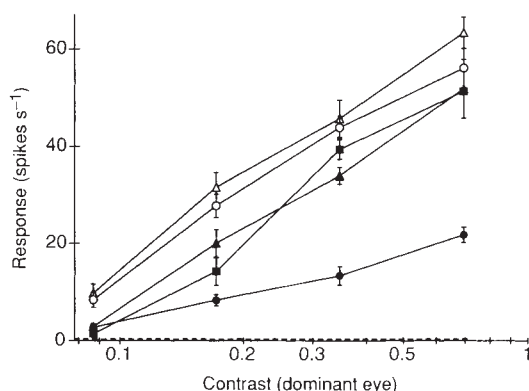


FIG. 3 Contrast-response functions, as in Fig. 2, for a complex cell recorded in layer 2/3 of a cat made esotropic (convergent squint) by section of the lateral rectus muscle of the right eye 10 days after birth, producing a squint of approximately 35°. This cell was exclusively driven through the non-squinting eye. Drifting gratings shown to the dominant eye were optimized for orientation and spatial frequency and varied in contrast, exactly as for Fig. 2, but the stimulus for the non-dominant, 'silent' eye (presented in a position corresponding to that of the receptive field in the dominant eye) was a drifting grating (contrast = 0.7) of the same orientation as the optimum grating presented to the normal eye. Even such a matched stimulus produced gain reduction if presented when the cell was already responding through the dominant eye (filled circles). All curves represent data from 5 trials (mean  $\pm$  s.e.m.). Results were essentially similar for three other neurons tested in full.

screens to both eyes (interleaved with other presentations). The sudden onset of an inappropriate stimulus in one eye reduced response gain by about 50% at each contrast level but only when the cell was already responding through the dominant eye (filled circles). None of the other functions differed consistently from each other, nor from either control condition. Results were essentially similar for a further three neurons tested in full.

ments, might account for the shifts of eye dominance experienced by some strabismic humans (those with alternating fixation). However, many strabismic humans habitually fixate with one eye and hold the other eye perceptually suppressed: in such cases cortical suppressive mechanisms might have become biased in favour of one eye.

Previous theories of binocular rivalry<sup>19-21</sup> suggest that it might be caused by reciprocal inhibition between monocularly driven neurons with left- and right-eye inputs. Although Varela and Singer<sup>22</sup> reported orientation-dependent suppression for some monocular cells in the lateral geniculate nucleus (LGN), which provides input to the cortex, we<sup>23</sup> and others<sup>24</sup> found such effects to be independent of relative orientation and weak. Moreover, suppression in the LGN occurs even with simultaneous onset of the binocular stimuli<sup>24</sup>. Therefore the interactions in the cortex probably depend on intracortical mechanisms, as do the gain changes underlying adaptation, which are not seen in the LGN<sup>4,6</sup>.

Although intrinsic horizontal excitatory connections predominantly link cortical neurons of similar orientation<sup>25,26</sup>, inhibitory connections appear to be more uniformly distributed across orientation and ocular dominance columns<sup>27,28</sup>, connecting areas of both similar and orthogonal orientation preferences. One possible implementation of interocular suppression is reciprocal inhibition between cells dominated by the two eyes, lying in neighbouring ocular dominance columns. While intracortical excitatory connections may serve to 'amplify' the responses of cortical neurons to input from the thalamus<sup>29</sup> and reinforce binocular facilitation for matched stimuli in normal animals, suppression resulting from inhibition between cells of opposite ocular dominance could provide a mechanism to veto signals from one eye under conditions that would lead to diplopia and confusion. □

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## Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle

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NITRIC oxide is the major endothelium-derived relaxing factor (EDRF)<sup>1–3</sup>, and it is thought to relax smooth muscle cells by stimulation of guanylate cyclase, accumulation of its product cyclic GMP, and cGMP-dependent modification of several intracellular processes<sup>4,5</sup>, including activation of potassium channels through cGMP-dependent protein kinase<sup>6,7</sup>. Here we present evidence that both exogenous nitric oxide and native EDRF can directly activate single  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$  channels ( $\text{K}_{\text{Ca}}^{+}$ ) in cell-free membrane patches without requiring cGMP. Under conditions when guanylate cyclase was inhibited by methylene blue, considerable relaxation of rabbit aorta to nitric oxide persisted which was blocked by charybdotoxin, a specific inhibitor of  $\text{K}_{\text{Ca}}^{+}$  channels. These studies demonstrate a novel direct action of nitric oxide on  $\text{K}_{\text{Ca}}^{+}$  channels.

To test the direct effect of nitric oxide (NO) on potassium channels and to distinguish it from the cGMP-dependent pathway, excised membrane patches from rabbit aortic smooth muscle cells (SMC) were exposed directly to solution in which NO was freshly dissolved. Figure 1 shows the direct effect of NO on single  $\text{K}_{\text{Ca}}^{+}$  in the absence of cGMP,  $\text{Mg}^{2+}$ , and ATP. NO rapidly increased single  $\text{K}_{\text{Ca}}^{+}$  channel activity measured as open channel probability ( $\text{NP}_o$ ) (Fig. 1, left panels). Corresponding traces of single-channel currents before and after application of NO are shown on the right. NO dissolved to give approximate concentrations of 0.2 to 10  $\mu\text{M}$  (the actual concentration applied to the membrane was less) activated channels in 19 out of 24 inside-out membrane patches and in 4 out of 6 outside-out membrane patches, showing that NO can affect  $\text{K}_{\text{Ca}}^{+}$  channels from both sides of the membrane. Figure 1b shows the result of a typical experiment in which a membrane patch was exposed to less than 1  $\mu\text{M}$  NO. This concentration of NO is close to the physiological concentration (0.85  $\mu\text{M}$ ) measured in smooth muscle cells of the rabbit aorta following stimulation of its endothelial cells<sup>8</sup>. At this concentration, NO caused a sig-

nificant  $2.51 \pm 0.20$  fold increase in  $\text{NP}_o$  (mean  $\pm$  s.e.,  $n=6$ ,  $P<0.01$ ). Single-channel current traces in the right panels of Fig. 1a and b show the appearance of long bursts of channel openings after NO application even at low levels of channel activity. This effect was typical, and in some experiments even longer bursts of openings of up to 20 s were observed, preceded occasionally by very long (5–10 s) 'silent' periods when the channel was closed. NO did not affect single-channel conductance.

The magnitude of channel activation by NO tended to increase at higher NO concentrations (Fig. 1c). Note also that the experiment in Fig. 1c was done with no EGTA or calcium added to the solutions to exclude any changes in channel activity resulting from possible effects of NO ( $>1 \mu\text{M}$ ) on the buffering of free  $\text{Ca}^{2+}$  by EGTA. In most experiments, after the peak increase in channel activity caused by membrane exposure to NO, the activity decreased but remained higher than control levels even after 20 min of washout (Fig. 1c). This suggests that NO might cause persistent modification of channel activity under these experimental conditions. To show that NO itself and not the products of its chemical degradation are responsible for channel activation, in 3 experiments the solution in which NO was dissolved was exposed to air for 10–15 min before the membrane patch was transferred into it. No changes in channel activity were observed, although subsequent exposure of the same patches to freshly dissolved NO increased channel activity (not shown).

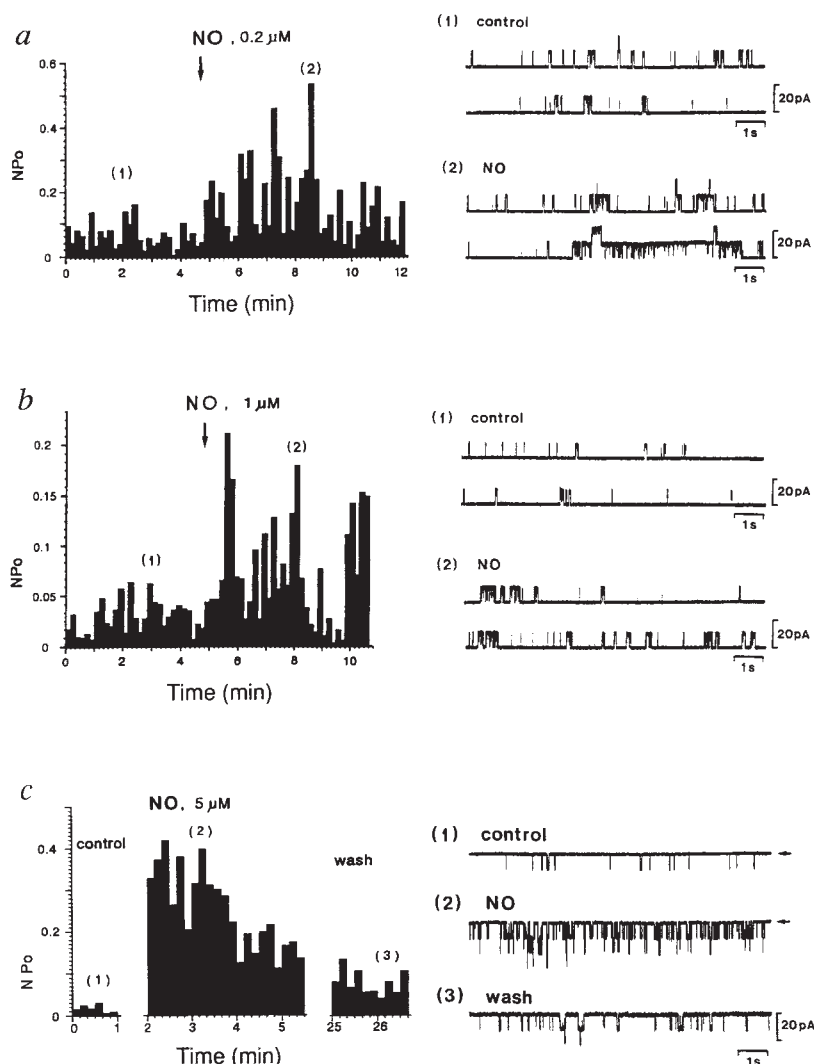
These results demonstrate that exogenous NO can activate  $\text{K}_{\text{Ca}}^{+}$  channels directly, in the absence of  $\text{Mg}^{2+}$ , guanine nucleotides or kinases known to be required for cGMP-dependent channel activation<sup>6,7,9</sup>. To determine if native EDRF which has been identified as  $\text{NO}^{1,10}$  can also activate  $\text{K}_{\text{Ca}}^{+}$  channels directly, outside-out membrane patches were exposed to perfusate coming out of a rabbit aorta. It has been demonstrated that ACh added to the lumen of rabbit aorta under similar conditions results in the continuous release of EDRF as evidenced by relaxation of arteries exposed to the perfusate<sup>10,11</sup>. Figure 2 shows a representative experiment in which an outside-out membrane patch was exposed directly to aortic perfusate before and during addition of ACh (1  $\mu\text{M}$ ) to the aortic lumen. Addition of ACh to the perfusate caused a significant increase in  $\text{NP}_o$  of the channels which averaged  $2.01 \pm 0.16$ -fold (mean  $\pm$  s.e.,  $n=5$ ,  $P<0.01$ ). In 3 control experiments ACh applied to the outside-out membrane patch without the aorta caused no change in channel activity.

To gain insight into the molecular mechanism by which NO activates  $\text{K}_{\text{Ca}}^{+}$  channels, we tested if chemical modification of sulphhydryl groups is involved as has been proposed for some proteins including albumin, tissue plasminogen activator, and the NMDA receptor<sup>12–14</sup>. *N*-ethylmaleimide (NEM) is known to covalently modify protein sulphhydryl groups making them incapable of nitrosylation<sup>12</sup>. NEM (5 mM) applied to excised membrane patches for 2 min produced  $3.08 \pm 1.00$  (mean  $\pm$  s.e.,  $n=5$ )-fold decrease in channel activity ( $P<0.1$ ) suggesting that covalent modification of sulphhydryl groups can alter normal channel activity. Pretreatment of the membrane patch with NEM (5 mM) prevented NO-induced activation of single  $\text{K}_{\text{Ca}}^{+}$  channels as shown in Fig. 3.  $\text{NP}_o$  during NO exposure after NEM was  $0.89 \pm 0.20$ -times (mean  $\pm$  s.e.,  $n=5$ ) the level during NEM pretreatment. This suggests that NO activation of channels is dependent on chemical modification of sulphhydryl groups present on the channel or other membrane constituents. Despite becoming insensitive to NO, the channels were still capable of being activated by fatty acid. In 3 experiments, application of myristic acid (40  $\mu\text{M}$ ) to the membrane pretreated with NEM produced a large increase in channel activity (Fig. 3). This indicates that NO modulation of channels differs from that caused by the fatty acid.

To determine if the direct cGMP-independent activation of  $\text{K}_{\text{Ca}}^{+}$  channels by NO is important in mediating smooth muscle relaxation, the effects of charybdotoxin (CTX) and the guanylate cyclase inhibitor, methylene blue (MB), were determined on NO-

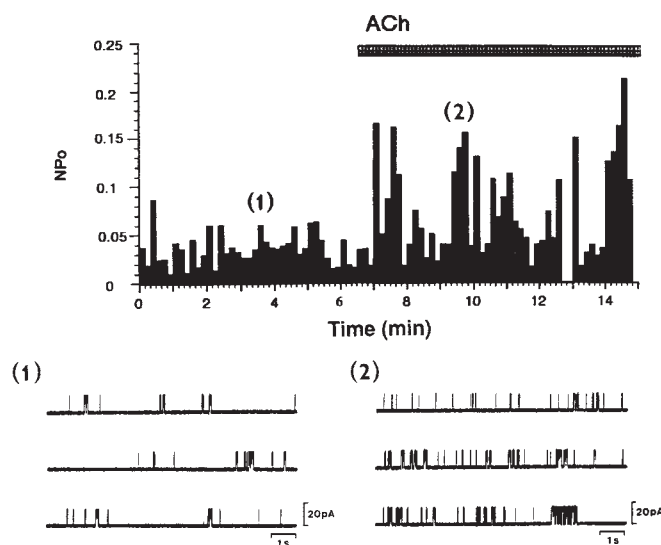
**FIG. 1** Nitric oxide (NO) directly activates single  $K_{Ca}^{+}$  channels in excised membrane patches in the absence of cGMP. *a, b*, The effect of 0.2  $\mu$ M (*a*) and 1  $\mu$ M (*b*) NO on single-channel activity of inside-out membrane patches. The left panels show the changes with time of channel activity (measured as open-state probability,  $NP_o$ ). The inner surface of the membrane was exposed to NO at the time indicated. On the right panels, traces of single channel currents are shown before (1) and after (2) NO application corresponding to the times indicated in the histograms on the left. Channel openings are upward deflections. Membrane potential +40 mV,  $[Ca]_{in} = 0.1 \mu$ M (*a*) and 0.2  $\mu$ M (*b*). *c*, The effect of NO (5  $\mu$ M) on single-channel activity in an inside-out membrane patch in 140 mM KCl (+10 mM HEPES) solution with no  $Ca^{2+}$  or EGTA added. In the left panel the channel activity (measured as  $NP_o$ ) is shown under control conditions (1), 1 minute after exposure to NO (2), and 20 minutes after NO was washed out completely (3). Single channel current traces on the right correspond to the times indicated in the histogram on the left. Channel openings are downward deflections. Membrane potential -40 mV, free  $[Ca]_{in} \sim 1-5 \mu$ M.

**METHODS.** Nitric oxide (NO) was prepared in water (4 °C) in air-tight Vacutainer tubes that were purged of  $O_2$  by bubbling with  $N_2$  for 60 min. NO gas was bubbled into 8 ml of water for 20 min to yield a saturated solution ( $\sim 1$  mM). The membrane patch was exposed to NO solutions by transferring the patch into the solution containing NO within 20–30 s after NO was dissolved there in concentrations stated in the text. It has been shown previously that in this period of time about half of the initial concentration of NO remains when a solution is exposed to air; after that, in the absence of biological tissue, the concentration decreases more slowly, and NO persists in solution for several minutes<sup>19</sup>. On the basis of these considerations, the concentrations of NO applied to the membrane in our experiments is actually less than that initially dissolved and stated in the text.



**FIG. 2** The direct effect of native EDRF on single  $K_{Ca}^{+}$  channels. An outside-out membrane patch was placed 3 mm from the outflow of a freshly isolated rabbit thoracic aorta perfused (1 ml min<sup>-1</sup>) with physiological solution (with 5.5 mM glucose added). The activity of  $K_{Ca}^{+}$  channels (measured as  $NP_o$ ) during the continuous exposure of a membrane patch to aortic effluent is shown before and after ACh (1  $\mu$ M), together with aortic perfusate, reached the patch (shown by the bar). Below, continuous traces of single-channel currents are shown at the times indicated on the histogram above. Channel openings are upward deflections. Membrane potential +40 mV,  $[Ca]_{in} = 0.2 \mu$ M.

**METHODS.** Single-channel currents were recorded using a standard patch-clamp technique<sup>20</sup>. Maxi- $K_{Ca}^{+}$  channels in our preparation were identified by their 250–300 pS conductance, the typical voltage and  $Ca^{2+}$ -dependency<sup>21–25</sup>, and their high sensitivity to charybdotoxin (CTX)<sup>26</sup>. Recordings were made using an Axopatch 200a amplifier. Data were filtered at 5–10 kHz, stored on tape, and later played back, digitized at 2–5 kHz, and computer analysed. Membrane patches with no more than 3 channels were used for experiments; channel number was not altered by NO. Channel activity was estimated by open-state probability,  $NP_o$ . The mean  $NP_o$  for 10-s intervals was plotted as histograms to show the changes in channel activity with time. The average levels of  $NP_o$  during 2–5 min of recording were estimated to show the major changes in channel activity following different treatments. The average  $NP_o$  or the fold changes in  $NP_o$  were expressed as mean  $\pm$  s.e.; *n* represents the number of individual patches tested under similar conditions. A Student's *t*-test was used to evaluate the statistical significance of changes in  $NP_o$ . For patch-clamp experiments SMC were placed in physiological solution (mM): NaCl 140, KCl 2.8, HEPES 10,  $CaCl_2$  1,  $MgCl_2$  2. The patch-pipette was filled with (in mM): KCl 140,



HEPES 10, EGTA 3 and different amounts of 200 mM stock  $CaCl_2$  added to give the final free  $Ca^{2+}$  concentration ranging from 0.1 to 1  $\mu$ M. The same pipette solution was used also in the bath in experiments with excised membrane patches. All the solutions were adjusted to pH = 7.4. Experiments were done at room temperature (20–22 °C).



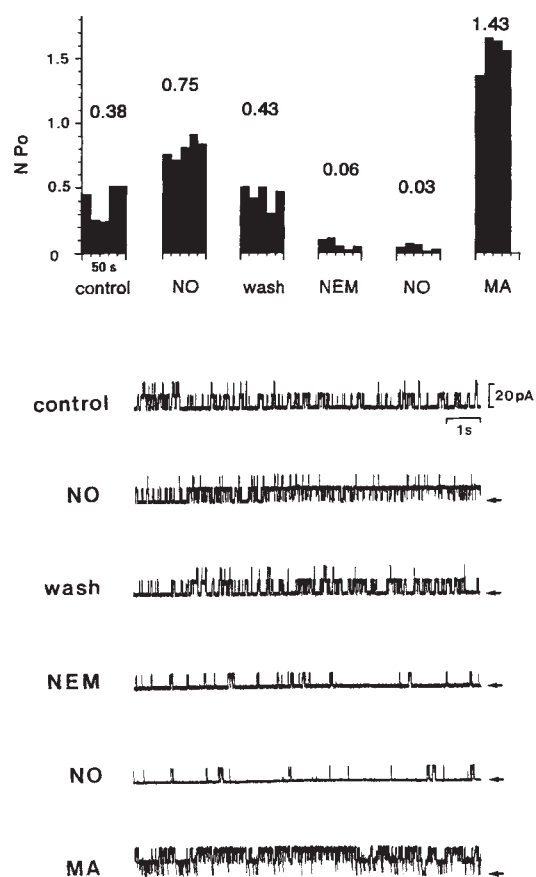
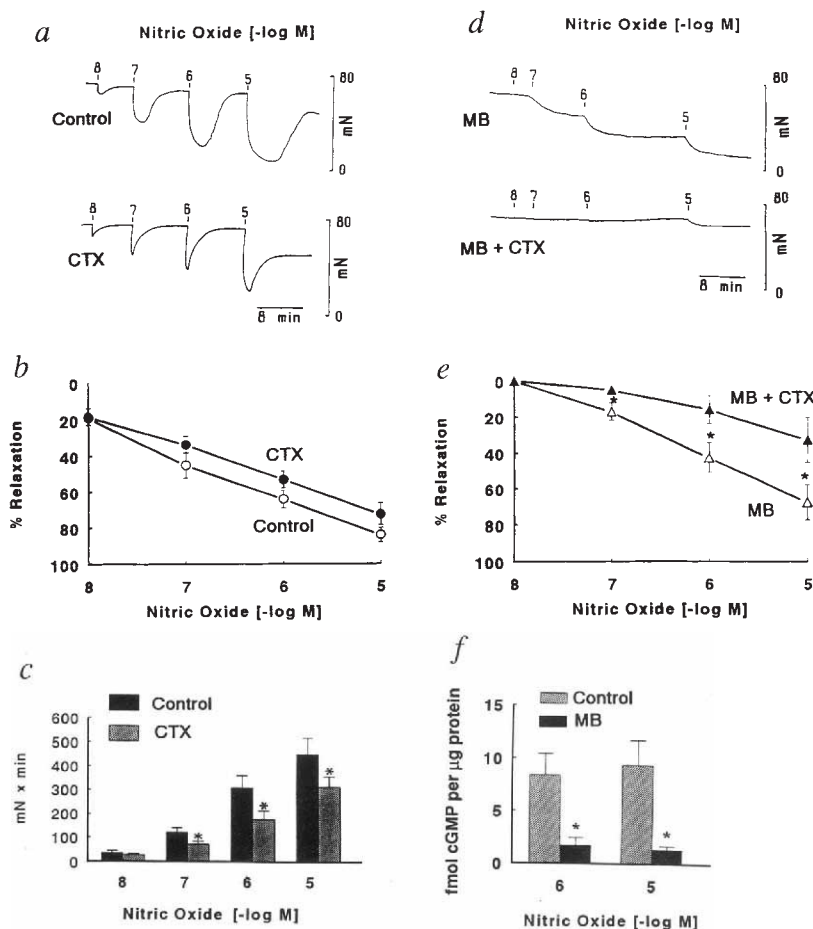


FIG. 3 Modification of sulphhydryl groups by *N*-ethylmaleimide (NEM) prevents the effect of nitric oxide (NO), but not of fatty acid. The upper panel shows  $NP_0$  in an outside-out membrane patch during 10-s intervals for a 50-s period: under control conditions, at the peak NO ( $1 \mu\text{M}$ ) effect, after 20 min of NO washout, at the end of 2 min application of NEM ( $5 \text{ mM}$ ), after subsequent addition of NO ( $1 \mu\text{M}$ ), and after subsequent application of myristic acid (MA,  $40 \mu\text{M}$ ) to the bath, respectively. Average  $NP_0$  for each period is indicated above. Corresponding single channel current traces are shown below. Openings are upward deflections. Membrane potential  $+40 \text{ mV}$ ;  $[\text{Ca}]_{\text{in}} = 0.5 \mu\text{M}$ . In the experiment shown, NO was applied only once before NEM, but in 3 other experiments NO repeatedly increased channel activity when applied (2–3 times, each for 2 min) to the membrane before NEM. The membrane patch was washed between NEM, NO and MA application.

**METHODS.** Single smooth muscle cells (SMC) from thoracic aorta of male New Zealand White rabbits were isolated by enzymatic dissociation and placed in primary culture for 2–12 days. The aorta was opened longitudinally and the intima was removed by careful scraping with a razor blade. Thin pieces of medial smooth muscle were gently stripped with forceps and placed for 100–120 min at  $37^\circ\text{C}$  in M199 containing 350 units  $\text{ml}^{-1}$  collagenase, 20 units  $\text{ml}^{-1}$  elastase, and  $2 \mu\text{l ml}^{-1}$  FBS. This procedure yielded dispersed cells, which were then pelleted (7 min at 500g). The pellet was resuspended in M199 containing 20% FBS, 2 mM glutamine, and  $1 \mu\text{g ml}^{-1}$  gentamycin. SMC were placed ( $3 \times 10^4$  cells  $\text{ml}^{-1}$ ) on coverslips in 35 mm dishes and were used for patch-clamp experiments after 2–12 days in primary culture. No significant differences in single  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel features were found throughout this time in primary culture.

FIG. 4 The effect of CTX alone, methylene blue alone, and their combination on NO-induced relaxation and cGMP level of rabbit aortic rings. **a**, Tracings of simultaneous relaxations to NO ( $10^{-8}$ – $10^{-5} \text{ M}$ ) of paired rings of rabbit thoracic aorta in the absence (control) or in the presence of CTX ( $50 \text{ nM}$ ). The ordinate is isometric tension and the abscissa is time. Matched contractions of the aortic rings were produced by adding phenylephrine ( $1\text{--}2 \times 10^{-7} \text{ M}$ ). A serially diluted saturated solution of NO was added to reach the final concentration indicated. Methods are described elsewhere<sup>27</sup>. Superoxide dismutase ( $150 \text{ units ml}^{-1}$ ) was added in all the experiments to prevent the oxidative breakdown of NO, as well as the scavenging of NO which has been demonstrated to occur in the presence of methylene blue<sup>28</sup>. **b**, Summary data showing the maximal level of relaxation reached following addition of each concentration of NO in 7 experiments, one of which is shown in **a**. Maximal relaxation was not significantly affected by CTX ( $50 \text{ nM}$ ). **c**, Histogram showing that the integrated time course of relaxation (mean  $\pm$  s.e.,  $n = 7$ ) in response to NO was significantly reduced by CTX ( $50 \text{ nM}$ ) in 7 experiments, one of which is shown in **a**. For each dose of NO, area above the relaxation curve and below the plateau established by the phenylephrine contraction was integrated in units of  $\text{mN} \times \text{min}$  for paired control and CTX-treated rings. The same period of time was integrated for both rings, from the moment of NO application to the time when relaxation reached a plateau in the ring treated with CTX. **d**, Tracings of simultaneous relaxations to NO ( $10^{-8}$ – $10^{-5} \text{ M}$ ) of aortic rings in the presence of methylene blue (MB,  $10^{-4} \text{ M}$ ) alone, or the combination (MB + CTX) of methylene blue ( $10^{-4} \text{ M}$ ) and charybdotoxin ( $50 \text{ nM}$ ). **e**, Cumulative data of maximal relaxation to NO of aortic rings treated with methylene blue ( $10^{-4} \text{ M}$ ) alone or together with CTX ( $50 \text{ nM}$ ) in 7 experiments. CTX significantly inhibited relaxations to NO ( $10^{-7}$ – $10^{-5} \text{ M}$ ) in the presence of methylene blue. **f**, Methylene blue ( $10^{-4} \text{ M}$ ) significantly reduced the content of cGMP in aortic rings exposed to NO ( $10^{-6}$ ,  $n = 7$ , and  $10^{-5} \text{ M}$ ,  $n = 6$ ). Rings were incubated



as described<sup>27</sup> and exposed for 1 min to NO. At the end of 1 min, each ring was frozen in liquid nitrogen and the cGMP content was determined by radioimmunoassay<sup>27</sup>.

induced relaxations of isolated rabbit aortic rings (Fig. 4). NO ( $10^{-8}$  to  $10^{-5}$  M) caused transient, concentration-dependent relaxations (Fig. 4a). CTX (50 nM) alone did not significantly affect the maximal relaxation caused by each concentration of NO (Fig. 4a, b), but did change the time course of relaxation (Fig. 4a), and significantly reduced the integrated relaxation (Fig. 4c). Inhibition of guanylate cyclase by methylene blue ( $10^{-4}$  M) caused a significant reduction in cGMP content of arterial rings stimulated with NO (Fig. 4f). However, significant relaxations of the rings to NO ( $10^{-7}$ – $10^{-5}$  M) persisted and became less transient in the presence of methylene blue (Fig. 4d). These methylene blue-resistant, NO-induced relaxations were significantly blocked by CTX (Fig. 4d, e). Thus, inhibiting guanylate cyclase with methylene blue unmasked cGMP-

independent relaxations to NO which are probably mediated by the direct effect of NO on  $K_{Ca}^{+}$  channels in the intact rabbit aorta.

In summary, this study demonstrates that both exogenous NO and native EDRF can directly activate  $K_{Ca}^{+}$  channels, which could cause hyperpolarization and relaxation of vascular smooth muscle<sup>15–17</sup> independently of cGMP. It was shown recently that ACh does not increase cGMP in atherosclerotic rabbit carotid arteries, but nevertheless causes normal relaxations which are inhibited similarly by CTX and an NO-synthase blocker<sup>18</sup>. Together with our present findings, these studies suggest that the NO-mediated cGMP-independent activation of  $K_{Ca}^{+}$  channels may circumvent the lack of the cGMP-dependent pathway to relax atherosclerotic arteries. □

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## Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons

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PHOSPHORYLATION of molecules involved in synaptic transmission by multifunctional protein kinases modulates both pre- and post-synaptic events in the central nervous system<sup>1,2</sup>. The positioning of kinases near their substrates may be an important part of the regulatory mechanism. The A-kinase-anchoring proteins (AKAPs; ref. 3) are known to bind the regulatory subunit of cyclic AMP-dependent protein kinase A with nanomolar affinity<sup>4–6</sup>. Here we show that anchoring of protein kinase A by AKAPs is required for the modulation of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainate channels<sup>4,5</sup>. Intracellular perfusion of cultured hippocampal neurons with peptides derived from the conserved kinase binding region of AKAPs prevented the protein kinase A-mediated regulation of AMPA/kainate currents as well as fast excitatory synaptic currents. This effect could be overcome by adding the purified catalytic subunit of protein kinase. A control peptide lacking kinase-binding activity had no effect. To our knowledge, these results provide the first evidence that anchoring of protein kinase A is crucial in the regulation of synaptic function.

Cyclic AMP and its principal target, cAMP-dependent protein kinase, regulate the release of transmitter as well as postsynaptic receptors and channels<sup>1–3,7–9</sup>. Although multiple effectors can elevate cAMP, a limited set of substrates are phosphorylated in response to activation by particular hormones or neurotransmitters. Several mechanisms have been proposed to account for this specificity<sup>10–15</sup>.

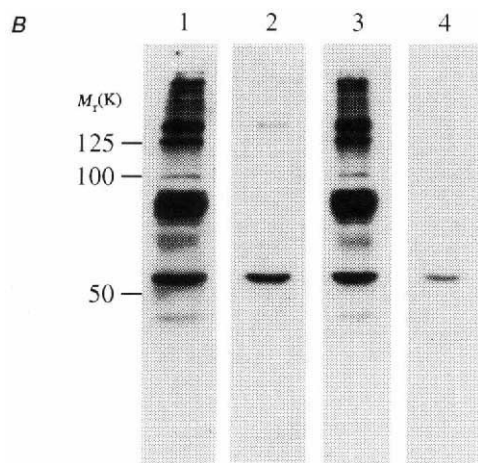
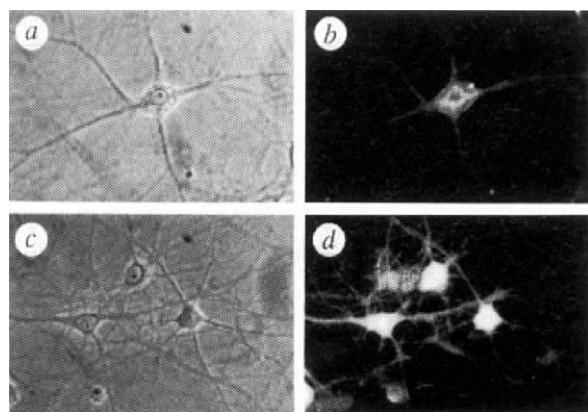
Biochemical and immunocytochemical analysis demonstrated that several AKAPs were present in cultured hippocampal neurons and were colocalized with the regulatory subunit RII of protein kinase A (PKA) in the soma and dendrites (Fig. 1A). The hippocampal neurons were immunoreactive when stained with antibodies prepared against RII (Fig. 1A, a and b) or AKAP79 (Fig. 1A, c and d), the human homologue of AKAP150 (ref. 3). Multiple RII-binding proteins with relative molecular masses of 50–200K were detected by the solid-phase binding of RII to AKAPs (Fig. 1B, lane 1). However, RII binding was blocked by preincubation with a 24-amino-acid peptide (Ht31 peptide<sup>6</sup>) derived from a conserved amphipathic helix common to the family of AKAP proteins (Fig. 1B, lane 2). The remaining 50K band was RII itself, as determined by western blots (Fig. 1B, lane 4). A 16-amino-acid control peptide derived from Ht31 peptide did not block binding of RII to AKAPs (Fig. 1B, lane 3). These peptides provide the reagents needed to test the functional consequences of A-kinase-anchoring in neurons.

PKA-dependent phosphorylation is required to maintain the function of AMPA/kainate channels in hippocampal neurons<sup>7,8</sup>. In the absence of ATP, whole-cell currents evoked by kainate (20  $\mu$ M) gradually declined over 25 min to  $50.9 \pm 5.2\%$  ( $n=8$ ) (Fig. 2b). This 'rundown' was prevented by addition of ATP ( $87.4 \pm 3.2$ ,  $n=12$ ,  $P<0.001$ ). The current also gradually decreased in the presence of ATP and the peptide PKI(5–24), a potent and specific inhibitor of the catalytic subunit of PKA ( $61.8 \pm 4.1\%$ ,  $n=11$ ,  $P<0.001$ ) (Fig. 2a, b). This confirmed that endogenous PKA modulates channel activity. To test the role of AKAPs in localizing PKA near the channel, anchoring inhibitor peptides were added to the whole-cell pipette. As shown in Fig.

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2, the anchoring inhibitor peptide derived from Ht31 (1  $\mu$ M) inhibited AMPA/kainate currents to the same extent as PKI ( $64.9 \pm 3.2\%$ ,  $n = 11$ ,  $P < 0.0001$ ). The effects of Ht31 peptide and PKI were not additive (Fig. 2c). These results indicate that PKA localization is required for the modulation of AMPA/kainate currents.

We considered that Ht31 peptide might compete with AKAPs, leading to a gradual displacement of type II PKA holoenzyme from the anchoring sites. Consistent with this, there was an initial delay in the action of the Ht31 peptide. Similar inhibition of AMPA/kainate currents was observed with a peptide derived from the RII binding region of AKAP79 (1  $\mu$ M,  $68.3 \pm 3.3\%$ ,  $n = 12$ ,  $P < 0.001$ ). In addition, a 16-amino-acid control peptide that does not bind RII (Fig. 1) had no effect on kainate currents ( $85 \pm 4.1\%$ ,  $n = 7$ ), indicating that the action of the anchoring inhibitor peptides was specific. The action of Ht31 peptide could be overcome by the catalytic subunit (0.3  $\mu$ M), suggesting that the anchoring inhibitor peptides interfered with PKA-dependent phosphorylation, but did not directly inhibit the kinase or channel gating. Currents evoked by AMPA (1  $\mu$ M,  $n = 6$ ) also ran down in the absence of ATP, and Ht31 peptide reduced the current ( $n = 6$ ), suggesting that the modulated channels were low-affinity AMPA receptors<sup>16</sup>. As low-affinity AMPA receptors are presumed to be heteromers of GluR1–4 which lack consensus PKA sites<sup>17–21</sup>, phosphorylation probably occurs at a 'non-consensus' site<sup>22</sup> or involves a regulatory protein near the channel.

Simulation of PKA by forskolin also enhances the synaptic currents mediated by AMPA/kainate receptors in hippocampal neurons<sup>8,23</sup>. To determine the effect of Ht31 peptide on spontaneous excitatory postsynaptic currents (e.p.s.cs) mediated by

FIG. 1 Expression of AKAPs in cultured hippocampal neurons. A, RII and AKAP 150 co-exist in the same cellular compartments of cultured hippocampal neurons. a, Phase contrast photomicrograph of a cultured hippocampal neuron; b, indirect immunofluorescence of the same cell incubated with affinity-purified RII antiserum (1:1,000 dilution). RII is excluded from the nucleus and is distributed in both the soma and neurites. c and d, Phase contrast photomicrograph and indirect immunofluorescence of neurons incubated with 1:1,000 AKAP 79 antiserum, the human homologue of murine AKAP 150 that is concentrated in postsynaptic densities<sup>3</sup>. Specificity of immunostaining with RII and AKAP150 antibodies was confirmed by the absence of fluorescence in parallel cultures incubated with nonimmune sera, and with the sera solid phase absorbed with homologous but not heterologous antigens (data not shown). Prominent staining of the soma and neurites suggests that RII and AKAP 150 are present in the same cellular compartments. B, Anchoring inhibitor peptides block the solid-phase binding of RII to AKAPs. Lane 1 shows the pattern of AKAPs expressed in cultured hippocampal neurons, where several prominent RII-binding bands ranging in size from 50K to 200K were detected. All RII-AKAP interactions were blocked when filters were incubated in the presence of 0.4  $\mu$ M Ht31 peptide (residues 494–515; lane 2), consistent with our previous observations<sup>6</sup>. In contrast, incubation of filters in the presence of a Ht31 control peptide (residues 494–509; lane 3) did not block the solid-phase binding of RII to AKAPs. The presence of RII in cultured hippocampal neurons was confirmed by western blot using affinity-purified antiserum (lane 4).

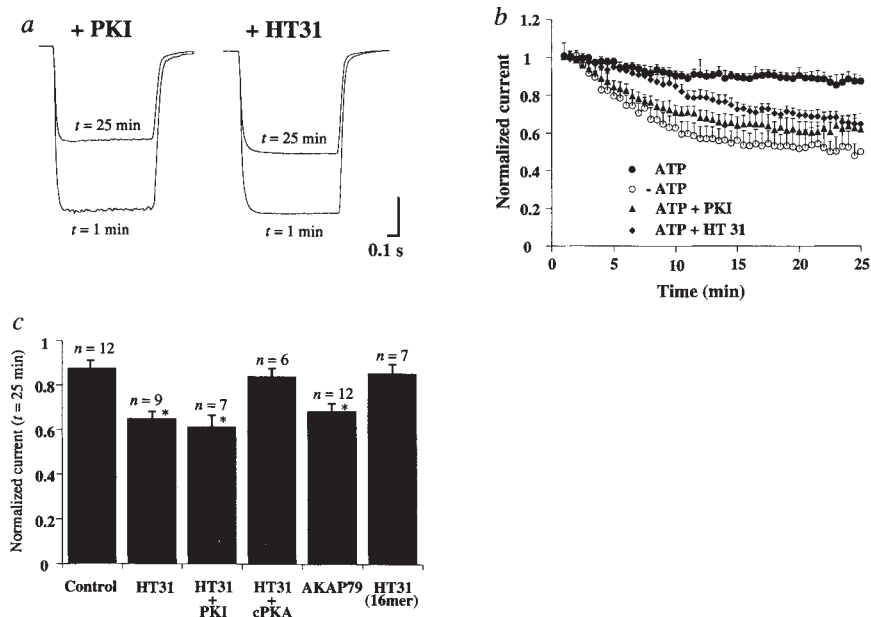
METHODS. Primary dissociated hippocampal cultures were prepared as described<sup>28</sup>. Hippocampal neurons, grown on coverslips for 7 days, were fixed with ice-cold methanol. Slides were washed for 1 h at room temperature in Tris-buffered saline/BSA/Brij 56 solution, then incubated overnight at 4 °C with primary antibodies. Immune complexes were tagged by incubation with FITC-conjugated goat anti-rabbit secondary antibodies (1:100 dilution) for 2 h at room temperature. Detection of fluorescence was by excitation at 470 nm on a Leitz fluorescent microscope. The presence of AKAPs and RII was investigated by the RII-overlay procedure for anchoring proteins<sup>6</sup>. Protein samples (50  $\mu$ g) were separated on a 10% SDS-polyacrylamide gel. Blots were incubated in a blotting solution (Tris-buffered saline (pH 7.4)/5% w/v dry milk/0.1% w/v BSA) containing purified murine RIIa (200 ng ml<sup>-1</sup>) and solid-phase RII was detected by western blotting with affinity-purified RII antibodies (1:2,000) using an ECL detection kit (Amersham).

AMPA/kainate receptors, spontaneous e.p.s.cs were analysed at the beginning of whole-cell recording and after 25 min of intracellular dialysis. In the presence of ATP, the amplitude of spontaneous e.p.s.cs was well maintained ( $90.4 \pm 4.7\%$ ;  $n = 7$ ), with no change in spontaneous e.p.s.cs frequency or duration. Ht31 peptide caused a significant decrease in spontaneous e.p.s.cs amplitude ( $73.9 \pm 3.7\%$ ;  $n = 6$ ,  $P < 0.02$ ) with no change in the decay of the current (Fig. 3a, b). As expected, there was no change in e.p.s.cs frequency, suggesting that Ht31 peptide in the postsynaptic cell did not affect release from the presynaptic terminal. As previously described<sup>8,23</sup>, forskolin (50  $\mu$ M,  $n = 5$ ) enhanced the spontaneous e.p.s.c. frequency ( $222 \pm 48\%$ ), presumably by a presynaptic mechanism, but produced only a small increase in amplitude ( $105 \pm 48\%$ ), indicating that endogenous activity of PKA was sufficient to maintain the synaptic AMPA/kainate channels. Thus AKAP-PKA interactions are also important in the regulation of synaptically activated AMPA/kainate channels.

These results offer physiological evidence that anchoring of kinases by AKAPs are critical in the phosphorylation of target proteins. AKAPs appear to be bifunctional molecules that have a conserved kinase-binding site, but unique targeting domains responsible for localizing AKAP-PKA complexes to cellular compartments<sup>3,5,24–26</sup>. The anchoring inhibitor peptides disrupted all PKA-AKAP interactions, thus the specific AKAP responsible for the regulation of AMPA/kainate channel activity remains to be determined. AKAP150 is one candidate as it is present in rat and mouse postsynaptic densities<sup>3</sup>. These results suggest that the local concentration of kinase is likely to be important in regulating PKA-dependent phosphorylation at the

FIG. 2 Displacement of RII subunit by anchoring inhibitor peptides blocks the regulation AMPA/kainate channels by PKA. **a**, Inward currents evoked by kainate (20  $\mu$ M) at 1 and 25 min after the start of whole-cell recordings are superimposed. Left, the current gradually decreased in presence of PKI(5–24) peptide (1  $\mu$ M) in the recording pipette. Right, a similar decrease in channel activity was seen during whole-cell dialysis with Ht31 peptide (1  $\mu$ M), a synthetic 24-amino-acid peptide derived from A-kinase-anchoring protein Ht31, which is a competitive inhibitor of RII anchoring. The holding potential was  $-60$  mV. ATP (5 mM) was included in the patch pipette. Calibration bar is 200 pA (left panel) and 360 pA (right panel). **b**, The time course of AMPA/kainate currents during whole-cell recording. Responses were normalized to the peak amplitude at 1 min after the start of recording. Symbols indicate mean  $\pm$  s.e. The loss of activity was similar in the absence of ATP (rundown) or when PKI or Ht31 peptide was added. The onset of action of Ht31 peptide was slightly delayed (see text). **c**, Amplitude of AMPA/kainate currents after 25 min of recording in the presence of 5 mM ATP in the patch pipette. The control and Ht31 peptide results are taken from **b**. Additional histogram bars show mean current amplitudes with the following reagents in the patch pipette: Ht31 peptide (1  $\mu$ M) and PKI (1  $\mu$ M); Ht31 peptide and catalytic subunit (cPKA, 0.3  $\mu$ M); AKAP 79 anchoring-inhibitory peptide (1  $\mu$ M); and a control peptide derived from Ht31 peptide that does not bind RII (Ht31, 16-mer). Asterisks indicate statistical significance reduction in current compared to control.

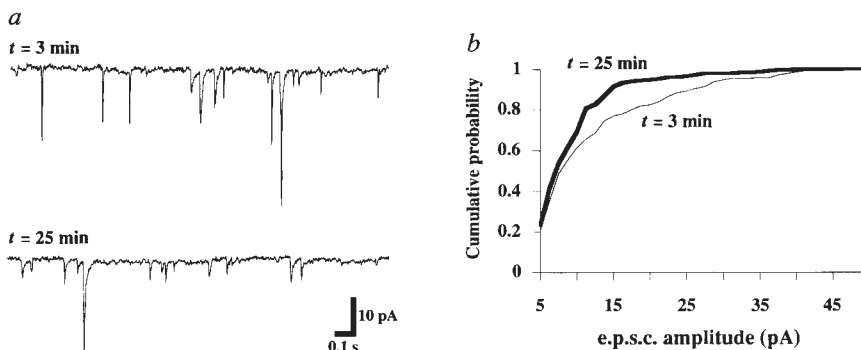
METHODS. Whole-cell recordings of neurons 5–12 days in culture were made with an Axopatch 1C amplifier (Axon Instruments). The intracellular medium under recording conditions contained: 165 mM NaCl, 2.4 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM glucose, 10 mM HEPES, 1  $\mu$ M tetrodotoxin (TTX), 100  $\mu$ M picrotoxin, pH 7.2; 325 mOsm. Recording pipettes had a resistance of 1–2 M $\Omega$  and contained 155 mM caesium gluconate, 10 mM HEPES, 10 mM BAPTA, 5 mM Mg-ATP, 2 mM  $\text{MgCl}_2$ , pH 7.3; 315 mOsm. Only neurons with series resistance of  $<8$  M $\Omega$  were included in the analysis. Series resistance (70–90%) and the cell capacitance were compensated and continuously moni-



tored during recording. Reagents were introduced into the cell by diffusion from the pipette; exchange times for small peptides was estimated to be 1–3 min based on test reagents<sup>29</sup>. AMPA/kainate channel activity was measured by applying agonist pulses at set intervals (0.3–1 s; every 30 s). Solutions were exchanged using a series of flow pipes (400  $\mu$ m internal diameter) positioned within 100–200  $\mu$ m of the neuron, and connected to gravity-fed reservoirs. Each flow pipe was controlled by solenoid valve and the assembly was moved with a piezoelectric bimorph (Vernitron, USA). Membrane currents were sampled at 1–2 kHz, filtered at 500 Hz and stored on an IBM-compatible computer using PClamp (V.5.5, Axon Instruments). For statistical analysis, the agonist-evoked membrane current at 1 min was compared with the response after 25 min of recording. Data are expressed as per cent of control  $\pm$  s.e. Significance was tested using analysis of variance with the Bonferroni–Dunn test for multiple comparisons; *P* values are given when significant.

FIG. 3 Dialysis with Ht31 peptide reduced the amplitude of spontaneous e.p.s.cs. **a**, Spontaneous AMPA/kainate-receptor-mediated e.p.s.cs at the beginning (3 min) and end (25 min) of the recording period at a holding potential of  $-60$  mV. **b**, Cumulative probability histogram of spontaneous e.p.s.c. amplitudes from the same neuron as in **a**. The amplitudes of spontaneous e.p.s.cs were reduced by Ht31 peptide (1  $\mu$ M). The threshold for peak detection of spontaneous e.p.s.cs for this neuron was set at 4 pA and data were binned at 2 pA intervals. The frequency of spontaneous e.p.s.cs did not change in the presence of Ht31 peptide. The mean frequency was 21 Hz at 3 min and 22 Hz at  $t = 25$  min.

METHODS. The extracellular solution contained TTX (1  $\mu$ M), 5 mM  $\text{Mg}^{2+}$  with no added glycine, picrotoxin (100  $\mu$ M) and strychnine (2  $\mu$ M) to block sodium channels, NMDA receptors and GABA/glycine channels, respectively. The peak amplitude and the half-width (duration at 50% of peak amplitude) for each spontaneous e.p.s.c. was measured using



AXOGRAPH software (Axon Instruments). Threshold for peak detection was set for each cell depending on the baseline noise and held constant throughout the recording period. Currents were acquired in 1-min epochs, filtered at 1–2 kHz (8-pole Bessel) and sampled at 2–4 kHz. Significance was tested using Student's *t*-test.

synapse. Given the presence of multiple phosphoproteins in the postsynaptic density and the existence of targeting proteins for other kinases<sup>27</sup>, compartmentalization of kinases may be a general mechanism for regulating the phosphorylation state of synaptic proteins, including receptors, enzymes and cytoskeletal elements. □

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## Antigen-specific human antibodies from mice comprising four distinct genetic modifications

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HUMAN sequence monoclonal antibodies, which in theory combine high specificity with low immunogenicity, represent a class of potential therapeutic agents. But nearly 20 years after Köhler and Milstein first developed methods for obtaining mouse antibodies<sup>1</sup>, no comparable technology exists for reliably obtaining high-affinity human antibodies directed against selected targets. Thus, rodent antibodies<sup>2</sup>, and *in vitro* modified derivatives of rodent antibodies<sup>3–5</sup>, are still being used and tested in the clinic. The rodent system has certain clear advantages; mice are easy to immunize, are not tolerant to most human antigens, and their B cells form stable hybridoma cell lines. To exploit these advantages, we have developed transgenic mice that express human IgM, IgG and IgK in the absence of mouse IgM or IgK. We report here that these mice contain human sequence transgenes that undergo *V(D)J* joining, heavy-chain class switching, and somatic mutation to generate a repertoire of human sequence immunoglobulins. They are also homozygous for targeted mutations that disrupt *V(D)J* rearrangement at the endogenous heavy- and  $\kappa$  light-chain loci. We have immunized the mice with human proteins and isolated hybridomas secreting human IgG $\kappa$  antigen-specific antibodies.

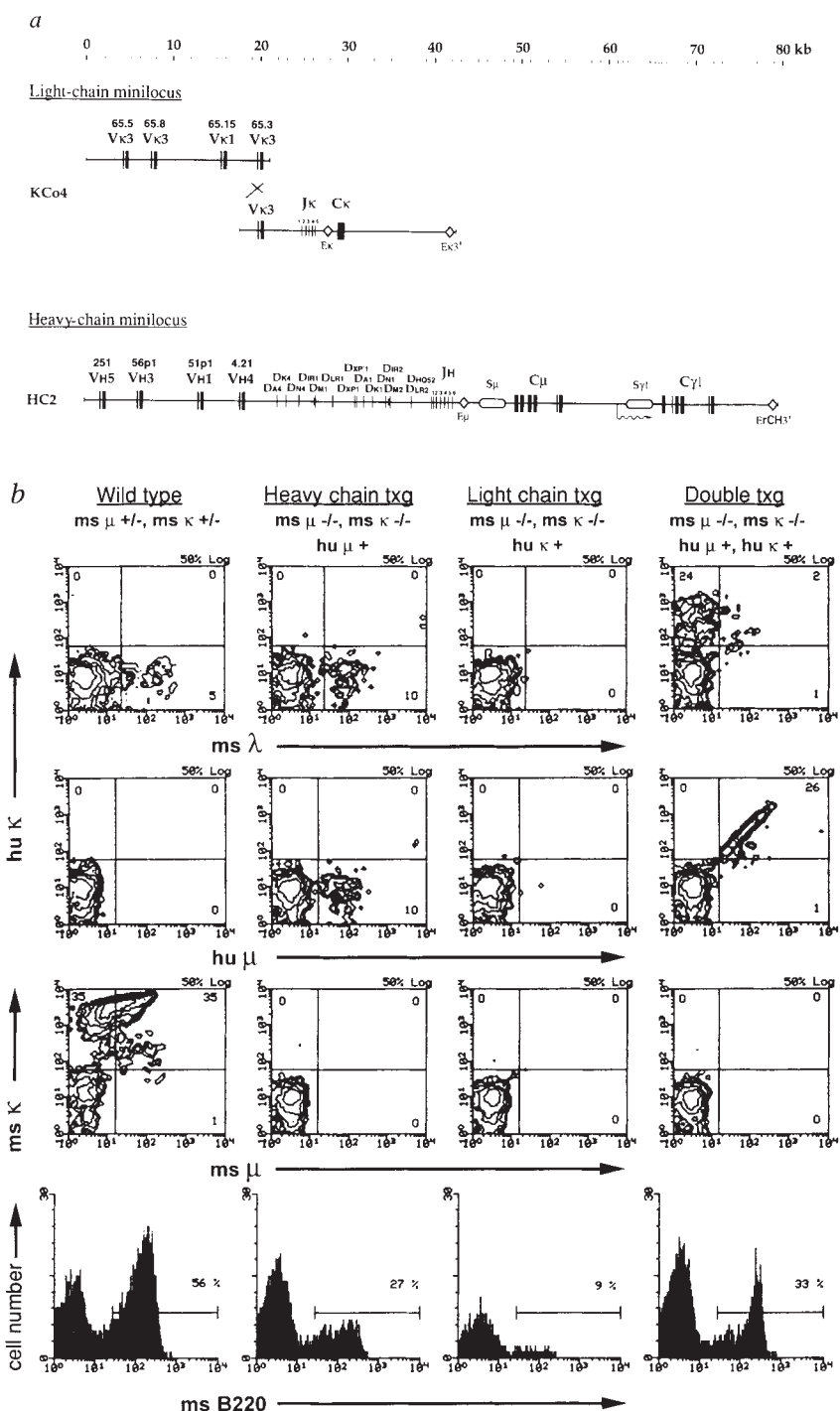
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FIG. 1 Human immunoglobulin heavy- and light-chain minilocus transgenes rescue the B-cell compartment of Ig<sup>-</sup> mutant mice. **a**, The KCo4 and HC2 transgene inserts are depicted before microinjection. The HC2 transgene is described elsewhere<sup>13</sup>. The light-chain minilocus, KCo4, is created by co-injection of two overlapping DNA fragments, each of which is propagated within a different plasmid. **b**, Flow cytometric analysis of fluorescent-stained cells isolated from spleens of 4 mice with different genotypes. For each of the 2-colour panels, the relative number of cells in each of the displayed quadrants is given as per cent of a 3-parameter, live-lymphocyte, gate based on propidium iodide staining and light scatter. The fraction of B220<sup>+</sup> cells in each of the samples displayed in the bottom row is given as a per cent of the lymphocyte light-scatter gate. Left column: control mouse (no. 9944, 6-week-old female JH<sup>+/+</sup>, JCK<sup>+/+</sup>). Second column: human heavy-chain transgenic (no. 9877, 6-week-old female JH<sup>+/+</sup>, JCK<sup>-/-</sup>, HC2 line 2550<sup>+</sup>). Third column: human  $\kappa$  light-chain transgenic (no. 9878, 6-week-old female JH<sup>+/+</sup>, JCK<sup>-/-</sup>, KCo4 line 4437<sup>+</sup>). Right column: double transgenic (no. 9879, 6-week-old female JH<sup>+/+</sup>, JCK<sup>-/-</sup>, HC2 line 2550<sup>+</sup>, KCo4 line 4437<sup>+</sup>). Top row: expression of mouse  $\lambda$  light chain (x-axis) and human  $\kappa$  light chain (y-axis). Second row: expression of human  $\mu$  heavy chain (x-axis) and human  $\kappa$  light chain (y-axis). Third row: expression of mouse  $\mu$  heavy chain (x-axis) and mouse  $\kappa$  light chain (y-axis). Bottom row: expression of mouse B220 antigen (log fluorescence: x-axis; cell number: y-axis). **c**, Comparison of pre-B- and B-cell numbers in wild-type (●) and double-transgenic/double-deletion (○) mice. Bone marrow, spleen and peritoneal cells isolated from three 3-month-old female double-transgenic/double-deletion (JH<sup>+/+</sup>, JCK<sup>-/-</sup>, HC2 line 2550<sup>+</sup>, KCo4 line 4437<sup>+</sup>) and 3 age-matched female wild-type B6CBF1 mice. The fraction of pre-B cells (B220<sup>+</sup>/IgM<sup>-</sup>) and B cells (B220<sup>+</sup>/IgM<sup>+</sup>) for each sample was determined on a wide scatter (including large and small lymphocytes, neutrophils and macrophages), propidium-iodide-negative gate. **d**, Secreted immunoglobulin levels in the serum of double-transgenic/double-deletion mice. Human  $\mu$ ,  $\gamma$  and  $\kappa$ , and mouse  $\gamma$  and  $\lambda$  from 18 individual HC2/KCo4 double-transgenic mice homozygous for endogenous heavy and  $\kappa$ -light chain locus disruptions. Mice (+) HC2 line 2550 (~5 copies of HC2 per integration), KCo4 line 4436 (1–2 copies of KCo4 per integration); (○) HC2 line 2550, KCo4 line 4437 (~10 copies of KCo4 per integration); (×) HC2 line 2550, KCo4 line 4583 (~5 copies of KCo4 per integration); (□) HC2 line 2572 (30–50 copies of HC2 per integration), KCo4 line 4437; (△) HC2 line 5467 (20–30 copies of HC2 per integration), KCo4 line 4437. **METHODS.** Construction of the light chain minilocus (KCo4): a V $\kappa$  specific oligonucleotide was used to probe a human placental genomic DNA phage library (Clontech). DNA fragments containing V $\kappa$  segments from positive phage clones were subcloned into plasmid vectors. Four different clones (v $\kappa$ 65.3, v $\kappa$ 65.5, v $\kappa$ 65.8 and v $\kappa$ 65.15) with open reading frames, intact splice acceptor and donor sequences, and intact recombination sequences were cloned together in a single plasmid, pKV4, with a 21-kb insert. The 3' V segment in this plasmid, v $\kappa$ 65.3, was also subcloned into a second plasmid together with a 22-kb  $\kappa$  constant region fragment that covers the  $\kappa$  constant region from 4.5 kb upstream of J $\kappa$ 1 to 13 kb downstream of C $\kappa$ . Transgenic mice: DNA fragments were isolated from plasmid vector DNA<sup>17</sup> by agarose gel electrophoresis and microinjected into the pronuclei of half-day (C57BL/6J × CBA/J)F<sub>2</sub> embryos<sup>18</sup>. Twenty-three light-chain-positive and 18

We constructed minilocus transgenes by stitching together non-contiguous genomic fragments containing functional coding and non-coding units of the human heavy- and  $\kappa$  light-chain immunoglobulin loci (Fig. 1a). The heavy-chain transgene, HC2, consists of an 80-kilobase (kb) DNA fragment containing four functional V segments, 15 D segments, 6 J segments, the  $\mu$  and  $\gamma$ 1 coding exons together with their respective switch regions, as well as the J $\mu$  intronic enhancer and the rat 3' heavy-chain enhancer<sup>6</sup>. All of the sequences are human except for the non-coding 3' enhancer.

The light-chain transgene, KCo4, is derived from the co-integration of two individually cloned DNA fragments at a single site in the mouse genome. The fragments together comprise four functional V $\kappa$  segments, 5 J segments, the C $\kappa$  exon, and both the intronic and downstream enhancer elements<sup>7,8</sup>. All of the sequences are of human origin. Because the two fragments share

heavy-chain-positive mice developed from injected embryos. Flow cytometry: bone marrow was isolated from both hind legs of each animal; spleen cells from entire spleen of each animal; and peritoneal cells from two consecutive 2 ml washes of peritoneal cavity from each animal. Red cells were lysed with  $\text{NH}_4\text{Cl}$  and total non-red-cell number determined using a Coulter counter (Coulter Electronics). Cell populations were determined using a FACScan flow cytometer and LYSIS II software (Becton Dickinson). Flow cytometry reagents: propidium iodide (Molecular Probes), phycoerythrin-conjugated anti-human and anti-mouse  $\text{Ig}\kappa$  (clone HP6062, Caltag; and clone X36, Becton Dickinson, respectively), FITC-conjugated anti-mouse  $\text{Ig}\lambda$  (clone R26 46) and  $\text{Ig}\mu$  (clone R6-60.2), and anti-human  $\text{Ig}\mu$  (clone G20-127), all from Pharmingen; Cy-Chrome and phycoerythrin-conjugated anti-mouse B220 (clone RA3-6B2; Pharmingen). *d*, Protein levels determined by ELISA. Human  $\mu$ : wells coated with mouse mAb anti-human  $\text{IgM}$  (clone CH6, The Binding Site, Birmingham, UK) and developed with peroxidase-conjugated rabbit anti-human  $\text{IgM}(\text{fc})$  (Jackson Immuno Research). Human  $\gamma$ : wells coated with mouse mAb anti-human  $\text{IgG1}$  (clone HP6069; Calbiochem) and developed with peroxidase-conjugated goat anti-human  $\text{IgG}(\text{fc})$  (Jackson). Human  $\kappa$ : wells coated with mouse mAb anti-human  $\text{Ig}\kappa$  (AMAC, Westbrook) and developed with peroxidase-conjugated goat anti-human  $\text{Ig}\kappa$  (Sigma). Mouse  $\gamma$ : wells coated with goat anti-mouse  $\text{IgG}$  (Jackson). Mouse  $\lambda$ : wells coated with rat mAb anti-mouse  $\text{Ig}\lambda$  (Pharmingen) and developed with peroxidase-conjugated rabbit anti-human  $\text{IgM}(\text{fc})$  (Jackson). Antibody standards: human  $\text{IgM}\kappa$  (Calbiochem), human  $\text{IgG1}\kappa$  (Calbiochem), mouse  $\text{IgG1}\lambda$  (Pharmingen), mouse  $\text{IgG}$  (Southern Biotechnology).





a common 3-kb sequence, they can integrate into the mouse genome as a contiguous 43-kb transgene following homologous recombination between the overlapping sequences<sup>9</sup>. Table 1 shows that the resulting miniloci from at least two of the transgenic lines rearrange to express a variety of light-chain transcripts. The success of the co-injection strategy is demonstrated by the expression of V segments from both fragments.

Heavy-chain and  $\kappa$  light-chain transgenic animals were bred together with strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy-chain and  $\kappa$  light-chain loci. The heavy-chain mutant strain, JHD, cannot undergo VDJ rearrangement because the J segments have been replaced with a neomycin-resistance gene<sup>10</sup>. These mice have no mature B cells. Similarly, the  $\kappa$  light-chain mutant mice, JCKD, cannot express  $\kappa$  chains as a result of deletion of the J $\kappa$  and C $\kappa$  gene segments<sup>11</sup>. The human sequence HC2 transgene rescues B-cell development in the JHD mutant background; all of the B220<sup>+</sup> cells in the periphery express cell-surface immunoglobulin receptors that use transgene-encoded heavy chain (Fig. 1b). The human KCo4 transgene is also functional, and competes successfully with the intact endogenous  $\lambda$  light-chain locus. Nearly 95% of the splenic B cells in KCo4/HC2 mice that are homozygous for both the JHD and JCKD mutations (double-transgenic/double-deletion mice) express completely human cell surface IgM $\kappa$ .

We find human IgM-expressing cells in the spleen, lymph-nodes, peritoneum and bone marrow of the double-transgenic/

TABLE 1 Human light-chain V and J segment usage in KCo4 transgenic mice

| Line | V $\kappa$ 65.5 | V $\kappa$ 65.8 | V $\kappa$ 65.15 | V $\kappa$ 65.3 | J $\kappa$ 1 | J $\kappa$ 2 | J $\kappa$ 3 | J $\kappa$ 4 | J $\kappa$ 5 |
|------|-----------------|-----------------|------------------|-----------------|--------------|--------------|--------------|--------------|--------------|
| 4436 | 0               | 11              | 4                | 3               | 14           | 1            | 0            | 2            | 1            |
| 4437 | 1               | 3               | 7                | 7               | 5            | 2            | 1            | 7            | 3            |

Indicated human  $\kappa$  sequences found in PCR clones, amplified from cDNA derived from two transgenic lines. cDNA was synthesized using spleen RNA isolated from two individual KCo4 transgenic mice (mouse 8490, 3 months old, male, KCo4 line 4437; mouse 8867, 2.5 months, female, KCo4 line 4436). The cDNA was amplified by PCR using a C $\kappa$  specific oligonucleotide, 5'-TAGAAGGAATTCAGCAGGCACACACAGAGGCAGTTCCA-3', and a 1:3 mixture of the following two V $\kappa$  specific oligonucleotides: 5'-AGCTTCTCGAGCTCCTGCTGCTGTTTCCAGGTGCC-3' and 5'-CAGCTTCTCGAGCTCCTGCTACTCTGGCTC(C,A)CAGATACC-3'. The PCR product was cloned into a plasmid vector, and partial nucleotide sequences determined by the dideoxy chain-termination method.

double-deletion mice. Although the peritoneal cavity contains the normal number of B cells, the absolute number of transgenic B cells in the bone marrow and spleen is typically 10–50% of normal (Fig. 1c). The reduction could be the result of a retardation in transgene-dependent B-cell development. This would have less impact on a self-renewing B-cell compartment, such as the peritoneum<sup>12</sup>, than on the bone marrow and spleen, which require a constant supply of newly formed cells.

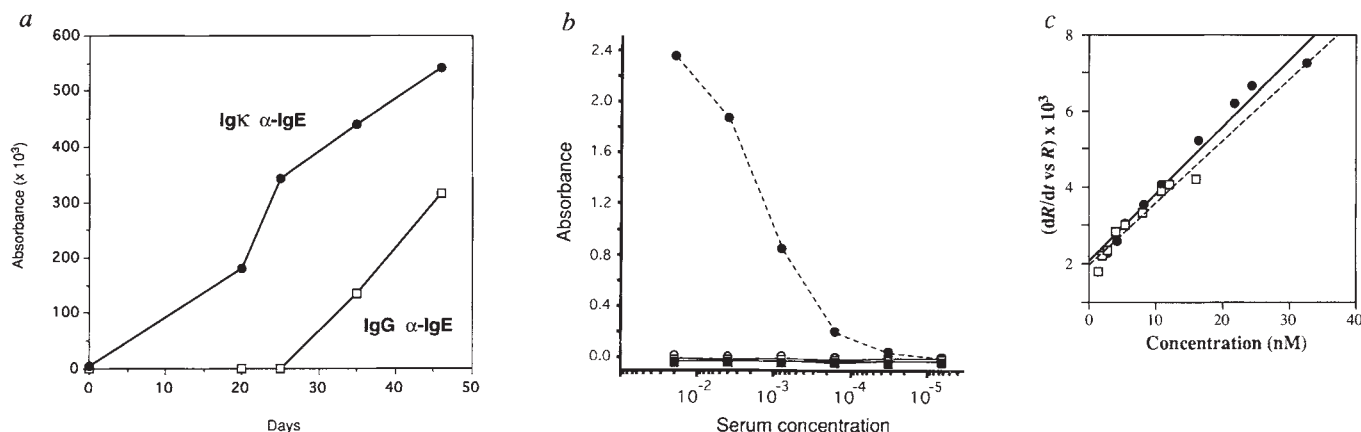


FIG. 2 Human antibody responses to human antigens. *a*, Time course of immune response. Antigen-reactive human IgG ( $\square$ ) and human  $\kappa$  light chain ( $\bullet$ ) in the serum of double-transgenic/double-deletion mouse given weekly injections of human IgE $\kappa$ . *b*, Titre and specificity of IgG response from double-transgenic/double-deletion mouse immunized with recombinant human soluble CD4. Human CD4-reactive human IgG was measured in diluted serum samples. The serum was also assayed for nonspecific reactivity to keyhole limpet haemocyanin (KLH). Human IgG anti-human CD4 (circles): pre-immunization ( $\circ$ ); post-immunization ( $\bullet$ ). Human IgG anti-human KLH (squares): pre-immunization ( $\square$ ); post-immunization ( $\blacksquare$ ). *c*, Determination of the binding constants for human IgG $\kappa$  anti-CD4 mAbs. The plot shows  $dR/dt$  versus  $R$  (change in response units over time versus response) plotted against the mAb concentration. The equations for the two curves ( $dR/dt$  versus  $R = k_{\text{ass}} \text{Conc} + k_{\text{diss}}$ ) are  $y = 1.63E + 05x + 1.95E - 03$  and  $y = 1.76E + 05x + 2.06E - 03$  for clones 2C5.1 ( $\square$ ) and 4E4.2 ( $\bullet$ ), respectively. The  $K_a$  values were obtained by dividing  $k_{\text{ass}}$  by  $k_{\text{diss}}$ .

**METHODS.** Mice homozygous for the human heavy-chain transgene HC2 and human  $\kappa$  light-chain transgene KCo4 were immunized with either *a*, 25  $\mu$ g human IgE $\kappa$  (The Binding Site), or *b*, 20  $\mu$ g recombinant human CD4 (American Bio-Tech.) in complete Freund's adjuvant on day 0 by intraperitoneal (i.p.) injection. Thereafter, animals were injected i.p. with antigen in incomplete Freund's adjuvant at approximately weekly intervals. *a*, Serum samples, collected at intervals over the course of the immunization, were diluted 1:10, and antigen-specific ELISAs per-

formed on human IgE $\lambda$ -coated plates (IgE $\lambda$  from The Binding Site). A representative result from one mouse (9344; HC2 line 2550, KCo4 line 4436) is shown. The human  $\kappa$  response correlates with the levels of human IgM in the serum of immunized animals. *b*, Serum from a representative animal (10068; HC2 line 2550, KCo4 line 4436), before and after 6 injections with human CD4. Human IgG was detected with peroxidase-conjugated polyclonal anti-human IgG (Jackson). Human  $\kappa$  light chain was detected using a peroxidase-conjugated polyclonal anti-human  $\kappa$  reagent (Sigma). *c*, Splenocytes from one of the CD4 immunized mice were isolated and fused with P3X63-Ag8.653 cells<sup>1</sup>. After an initial round of screening to detect anti-CD4 human IgG $\kappa$  mAbs by ELISA, cells from positive wells were subcloned. The subclones were shown by ELISA to be non-reactive with a panel of other antigens, including KLH, BSA, human serum albumin, carcinoembryonic antigen and ovalbumin. The CD4 binding affinities of two of the mAbs from this fusion were determined using a BiAcCore analyser (Pharmacia Biosensor). Human recombinant CD4 was covalently coupled through amine groups to the sensor chip surface, and binding measured for dilutions of the human IgG $\kappa$ -anti-CD4 mAb containing supernatants according to manufacturer's instructions. The amount of human IgG $\kappa$  mAb present in the supernatants was determined in a quantitative ELISA using mouse mAb anti-human IgG1 as the capture reagent, peroxidase conjugated mouse anti-human Ig $\kappa$  as the detecting reagent and human IgG1 $\kappa$  as the standard.

TABLE 2 The variable regions of human  $\gamma$  transcripts in HC2 transgenic mice contain non-germline-encoded nucleotides

| VH segment | Number of clones | Number of non-germline-encoded nucleotides | Frequency of non-germline-encoded nucleotides (%) |
|------------|------------------|--------------------------------------------|---------------------------------------------------|
| VH251      | 0                | —                                          | —                                                 |
| VH56P1     | 19               | 100                                        | 2.1                                               |
| VH51P1     | 1                | 5                                          | 2.0                                               |
| VH4.21     | 3                | 0                                          | 0.0                                               |

23 cDNA clones from an HC2 transgenic mouse were partially sequenced to determine the frequency of non-germline-encoded nucleotides within the variable regions. The data include only the sequence of V segment codons 17–94 from each clone, and does not include N regions. RNA was isolated from the spleen and lymph node of mouse 5250 (HC2 line 2550 hemizygous, JHD homozygous). Single-stranded cDNA was synthesized and  $\gamma$  transcripts amplified by PCR as described<sup>13,17</sup>. The amplified cDNA was cloned into plasmid vectors, and 23 randomly picked clones were partially sequenced by the dideoxy chain-termination method. The frequency of PCR-introduced nucleotide changes is estimated from constant-region sequence as <0.2%.

The double-transgenic/double-deletion mice also express fully human antibodies in the serum (Fig. 1d). We find significant levels of human  $\mu$ ,  $\gamma 1$ , and  $\kappa$  in these animals. In a separate report, we have shown that the expressed human  $\gamma 1$  results from authentic class switching by genomic recombination between the transgene  $\mu$  and  $\gamma 1$  switch regions<sup>13</sup>. Furthermore, we have found that intra-transgene class switching is accompanied by somatic mutation of the heavy-chain variable regions (Table 2).

In addition to human immunoglobulins, we find mouse  $\gamma$  and  $\lambda$  in the serum of these mice. The presence of mouse  $\lambda$  protein is expected because the endogenous locus is completely intact. The mouse  $\gamma$  expression appears to be a consequence of trans-switch recombination of transgene *VDJ* segments into the endogenous heavy-chain locus<sup>13</sup>. Trans-switching, which was originally observed for wild-type heavy-chain alleles and rearranged *VDJ* transgenes<sup>14,15</sup>, occurs in the mutant JHD background because the downstream heavy-chain constant regions and their respective switch elements are still intact.

To test the ability of the transgenic B cells to participate in an immune response, we immunized the mice with human protein antigens, and monitored serum levels of antigen-specific immunoglobulins. The initial human antibody response is IgM, followed by the emergence of antigen-specific human IgG (Fig. 2a). The lag before the appearance of IgG antibodies is consistent with an association between class switching and a secondary response to antigen. In a transgenic mouse immunized with human CD4, human IgG reactivity to antigen is detectable at serum concentrations ranging from  $2 \times 10^{-2}$  to  $1.6 \times 10^{-4}$  (Fig. 2b).

We isolated hybridoma cell lines from one of the mice that responded to human CD4 immunization. Five of the cloned hybridomas secrete human IgG $\kappa$  antibodies that bind to recombinant human CD4 and do not crossreact (as tested by ELISA) with a panel of other glycoprotein antigens. We determined the association and dissociation rates of the immunizing CD4 antigen for two of these antibodies (Fig. 2c). The experimentally derived binding constants ( $K_a$ ) are about  $9 \times 10^7$  and  $8 \times 10^7$  M<sup>-1</sup> for antibodies 4E4.2 and 2C5.1, respectively. These  $K_a$  values fall within the range of murine IgG anti-CD4 antibodies that have been used in clinical trials<sup>16</sup>.

We conclude that human immunoglobulin-expressing B cells undergo normal development, and respond to antigen, in the context of a mouse immune system. Antigen responsiveness leads to immunoglobulin heavy-chain isotype switching and variable region somatic mutation. We have also demonstrated that conventional hybridoma technology can be used to obtain human sequence antibodies from these mice. Therefore, these animals

represent a source of human antibodies against human target antigens. □

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## Cloning and functional expression of a cyclic-nucleotide-gated channel from mammalian sperm

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CYClic nucleotide-gated (CNG) channels serve as downstream targets of signalling pathways in vertebrate photoreceptor cells and olfactory sensory neurons (see ref. 1 for review).  $\text{Ca}^{2+}$  ions that enter through CNG channels<sup>2–5</sup> intimately control these signalling pathways by regulating synthesis<sup>6</sup> or hydrolysis<sup>7</sup> of cyclic nucleotides, and by decreasing ligand sensitivity of CNG channels<sup>8</sup>. Several lines of evidence suggest that cyclic nucleotides and  $\text{Ca}^{2+}$  play important roles in chemotaxis of invertebrate sperm and fertilization (see ref. 9 for review), whereas their mechanisms of action in vertebrate sperm are largely unknown. Here we report the cloning and functional expression of a novel CNG channel from bovine testis. The channel polypeptide was functionally localized in sperm, but is also specifically expressed in cone photoreceptor cells. These channels might be involved in chemotaxis of sperm by controlling  $\text{Ca}^{2+}$  entry through a cyclic-nucleotide signalling pathway.

We isolated several partially overlapping complementary DNA clones from a bovine testis cDNA library (see legend to Fig. 1). These clones exhibited a high degree of sequence similarity to known CNG channel sequences. The complete cDNA sequence of the recombinant clone pCGTE encodes a protein of 706 amino-acid residues with a calculated relative molecular mass ( $M_r$ ) of 81,132. The testis CNG channel shows the highest amino-acid sequence similarity with the cone photoreceptor channel from chicken<sup>10</sup> (78% identical residues).

The testis CNG channel contains all sequence motifs that are characteristic of this family of ion channels: the cyclic



nucleotide-binding site<sup>11</sup>, the pore region<sup>12</sup> and six putative transmembrane segments, including a voltage-sensor-like S4 motif<sup>13</sup> (Fig. 1).

Figure 2a shows a series of macroscopic current-voltage ( $I$ - $V$ ) recordings ( $I_{\text{mean}} = 533 \pm 477$  pA; range 60–1,500 pA at +80 mV,  $n = 10$ ) from inside-out patches of *Xenopus* oocytes injected with channel-specific cRNA. Maximal currents  $I_{\text{max}}$ , activated by saturating cyclic AMP concentrations were  $\leq 30\%$  of  $I_{\text{max}}$  activated by saturating cGMP (Fig. 2a, inset). The dependence of  $I/I_{\text{max}}$  on the cGMP and cAMP concentrations are shown in Fig. 2b. Mean values for half-maximal activation ( $K_{1/2} \pm \text{s.d.}$  (number of experiments) at  $V_m = +80$  mV) were  $8.3 \pm 2.1$   $\mu\text{M}$  ( $n = 10$ ) for cGMP and  $1,720 \pm 456$   $\mu\text{M}$  ( $n = 7$ ) for cAMP. The Hill coefficients were  $2.6 \pm 0.4$  (cGMP) and  $1.5 \pm 0.2$  (cAMP).

The ion selectivity of the channel was determined by measuring the reversal voltage  $V_{\text{rev}}$  under symmetrical bi-ionic conditions (Fig. 2c). Permeability ratios  $P_i/P_K$ , calculated from  $V_{\text{rev}}$  (corrected for the liquid junction potentials) according to the Goldman-Hodgkin-Katz equation yielded the following series of ion selectivity:  $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ \geq \text{Rb}^+ > \text{Li}^+ > \text{Cs}^+$  in the proportions 3.8:1:0.78:0.76:0.67:0.49. The single-channel conductance at +60 mV was 21 pS (Fig. 2d).

We identified functional CNG channels *in situ* by measuring cGMP-induced  $\text{Ca}^{2+}$  influx using Fura-2 fluorescence imaging. The method was developed and validated using HEK 293 cells transfected with CGTE cDNA. Application of 1 mM 8Br-cGMP

increased the intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ), up to 10-fold in transfected (Fig. 3a) but not in mock-transfected HEK 293 cells. The cGMP-induced rise in  $\text{Ca}^{2+}$  ( $\Delta[\text{Ca}^{2+}]_i$ ) could be repetitively evoked when cells had recovered from each preceding stimulation after washout of cyclic nucleotides. When 1 mM 8Br-cAMP was used, the rate of  $\text{Ca}^{2+}$  influx was 9.7% (s.e.m., 0.8%,  $n = 8$ ) of that induced by 1 mM 8Br-cGMP.

Extracellular  $\text{Mg}^{2+}$  ions reversibly inhibited the cGMP-induced  $\Delta[\text{Ca}^{2+}]_i$  in a cooperative fashion (half-maximal inhibition at  $31 \pm 4.2$   $\mu\text{M}$   $\text{Mg}^{2+}$ ; Hill coefficient of 1.8;  $n = 5$ ; Fig. 3a). This observation is consistent with the high sensitivity of the heterologously expressed bovine rod CNG channel to cooperative blockage by external  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (ref. 14).

Attempts to detect a cGMP-induced  $\Delta[\text{Ca}^{2+}]_i$  in sperm by Fura-2 imaging were unsuccessful. The nucleus occupies most of the cell volume and readily accumulates Fura-2 dye. Thus,  $\Delta[\text{Ca}^{2+}]_i$  might not be detectable in the presence of a large background fluorescence. However, one distinct population of membrane vesicles responded to 8Br-cGMP with a  $\Delta[\text{Ca}^{2+}]_i$ . These vesicles were spherical (2–4  $\mu\text{m}$  in diameter), characterized by a single membraneous appendage ('cap' vesicles), and did not contain a nucleus. They might represent cytoplasmic droplets from sperm. Roughly 10% of the vesicles responded to 8Br-cGMP.  $[\text{Ca}^{2+}]_i$  increased by 237% (s.e.m., 36%,  $n = 24$ ) with 1 mM 8Br-cGMP, and by 157% (s.e.m., 46%;  $n = 10$ ) with 1 mM 8Br-cAMP.

When  $\text{Ca}^{2+}$  was removed from the superfusing medium, 8Br-

FIG. 1 Amino-acid sequence alignment of different CNG channels. BOVTESTIS, bovine testis channel; CHICKCONE, chicken cone channel<sup>10</sup>; BOVROD, bovine rod channel<sup>11</sup>; BOVOLF, bovine olfactory epithelium channel<sup>24</sup>. Numbers of amino-acid residues are given on the right. Identical residues with respect to the testis channel sequence are represented by a space; conserved residues by dots; gaps by dashes; putative functional domains by lines above the sequence: H1–H5, putative membrane-spanning regions; S4, voltage-sensor-like motif.

**METHODS.** We used degenerate oligonucleotide primers corresponding to the amino-acid sequences MISNMN (residues 397–402 of BOVROD) and MYLIK (residues 514–518) to amplify a fragment from bovine testis cDNA. The resulting PCR fragment was used to screen an unamplified oligo(dT)-primed cDNA library from bovine testis. A partial cDNA-clone was isolated and used to screen a mixture of an oligo(dT)-primed and a randomly primed cDNA library. Clone pH0-B2 (~2.3 kb) was isolated, missing the translational initiation site. A 5'-terminal restriction fragment of pH0-B2 was used to rescreen the library for additional overlapping clones. Thereby clone pH0-6.2 (0.65 kb) was isolated which contains cDNA extending beyond the 5' end of the coding region. Clone pH0-6.2 was cleaved with *Bsp*MI and *Eco*RV and ligated with the ~2 kb *Bsp*MI–*Not*I (filled in) fragment of clone pH0-B2 to yield the recombinant pCGTE carrying the complete cDNA sequence. Standard procedures were used.

|           |                                                                                        |     |
|-----------|----------------------------------------------------------------------------------------|-----|
| BOVTESTIS | MAK--ISTQYSHPTRTHPVSRVTRMDRLDCIENGL--SRTHLPC--EETSSSELQEGIAMETRGALAES--RQSSFTSQGP--TRL | 73  |
| CHICKCONE | M--N H Y *MHGL T E *R F-I SL -- RV * H *G -QT * R *M                                   | 74  |
| BOVROD    | MK VI N WH FVNI-NUVGPDP...TR AC SFSG--D *D *S *F ESET NPHAR F SN *H GQ SQ E            | 77  |
| BOVOLF    | M *EKAN *VKS P *NNHHHAP *AI *ASGKD *HRAS *QS *AA-- *LAE *----- *QQR *FR *              | 69  |
| BOVTESTIS | SRLLISLRAW SARHLHQEDQRPDSFLERF GAELQEVSSRESHVQFNVGSGEPDRGRSAWPLARNNTNTNNSEKDDKA        | 153 |
| CHICKCONE | F * * * H I * V * N *RSF * IR Q GGVN * F V FS * *E K E                                 | 154 |
| BOVROD    | QY ----- *G * LF V NSS K Q *P E                                                        | 101 |
| BOVOLF    | * *GV E *Y NF * *P * HT *Q *----- *DGKGD                                               | 116 |
| BOVTESTIS | KKEEKEEKEEKENPKKEEKK-----DSVMDPSSNNMYHWTAVPVFNWCLL                                     | 205 |
| CHICKCONE | E K VKEEKEEKEE K E DDKKDDKDDKDDKKEEQK-----VF * N * A                                   | 228 |
| BOVROD    | KK KE KS P *KNE *K DPEKKKKKEKDKKKKEEKK *V * T N FC * *M T *                            | 181 |
| BOVOLF    | DG KGKTKK F----- *LF * *W * F * L                                                      | 158 |
| BOVTESTIS | VCRACFDELQSEHLMLVLVDYSADILYGMMLVVRARTGFLEQGLMVMDASRLWKHYTQLHFKLDVL SLVPTDLAYFKL        | 285 |
| CHICKCONE | F * * * I * K F C * VF F F * K EK * RD *Q * L                                          | 308 |
| BOVROD    | *A *Y EY AF L * L F * * K *ER * ID * KS FQ * * L I F                                   | 261 |
| BOVOLF    | A S * KGY * V * IA *F * L * K *K * RDN IH *Q A * I A *                                 | 238 |
| BOVTESTIS | GMNYPFLRFNRLKLARLFEFFDRTETRTNYPNMFIRGNLVYLIIHWNACIYFAISKVFEGTDSWVYPNVSNPEYG            | 365 |
| CHICKCONE | * * * * * * * * * * * * * * * * * * I                                                  | 388 |
| BOVROD    | W * L * * * * * * * * * * * A N * * N *                                                | 341 |
| BOVOLF    | *HN * * * * S * * * * S V * * *                                                        | 318 |
| BOVTESTIS | RLSRKYIYSLWSTLTLTIGETPPPVKDEYLFVVIDFLVGVLFATVGNVGSMSNMNASRAEFQAKIDSIKQYMQF             | 445 |
| CHICKCONE | * * * * * S F A * * * * * H                                                            | 468 |
| BOVROD    | * * * * * F * * * * * H                                                                | 421 |
| BOVOLF    | Y E C * * * * * H                                                                      | 398 |
| BOVTESTIS | RKVTLDLETRVIRWFDYLWANKTKTVDKEVLKSLPKLKAIEIAINVHDLTRKVRIFQDCEAGLLVELVLKLRPAVFSFG        | 525 |
| CHICKCONE | N * K * * * * N * * * * A * Q * *                                                      | 548 |
| BOVROD    | * * * * * N A * S * * * Q *                                                            | 501 |
| BOVOLF    | * * * * * N A * S * * * Q *                                                            | 478 |
| BOVTESTIS | DYICKKGDIGREMYIIEGKLAWAEDGITQFVVLGDGSGYFGEISILNKGKSGNRRRTANIRSIGYSDLFCLSKDDLME         | 605 |
| CHICKCONE | * * * * * * * * * * * * * * * * *                                                      | 628 |
| BOVROD    | * * * * * * * * * * * * * * * * *                                                      | 581 |
| BOVOLF    | * * * * * A * A C * M *                                                                | 558 |
| BOVTESTIS | ALTEYPEAKKALEEKGRIIMKDNLIDEELAKAGADPKDIEKEVHELTSLDSLQTRFARLLAEYNATQMKVKRLSQL           | 685 |
| CHICKCONE | * * * * * A * * * * * S * Q * R *                                                      | 708 |
| BOVROD    | * * GM * * G * I * N * * T * * L * * M Q * * K *                                       | 661 |
| BOVOLF    | * * -V * * -G * -EVA * M -V * * Q * N * Y * T * Q * -V *                               | 637 |
| BOVTESTIS | SQVKMGLPPDGDAPQTEASQP-----                                                             | 706 |
| CHICKCONE | *R KYG * SLSV * * EK EEQKDD--                                                          | 735 |
| BOVROD    | KF * PL * D * FS I * S * * * * GPTDSTQD                                                | 690 |
| BOVOLF    | *K * QNNE * L * DGM * * * PAEKP---                                                     | 663 |

cGMP did not induce a  $\Delta[\text{Ca}^{2+}]_i$  ( $n=2$ ; Fig. 3c). This result suggests that  $\Delta[\text{Ca}^{2+}]_i$  does not originate from intracellular  $\text{Ca}^{2+}$  stores but from  $\text{Ca}^{2+}$  influx through CNG channels. Roughly 10-fold higher  $\text{Mg}^{2+}$  concentrations were required to inhibit  $\text{Ca}^{2+}$  flux into 'cap' vesicles ( $K_i \sim 300 \mu\text{M}$ ; Hill coefficient of 1.8;  $n=3$ ; Fig. 3b, d) compared with HEK 293 cells expressing the CNG channel. The difference is at least partially accounted for by the threefold higher external  $\text{Ca}^{2+}$  concentration used in the 'cap' vesicle experiments (legend to Fig. 3).

Small cGMP-activated currents ( $\sim 1 \text{ pA}$ ) were detected in 8 of 18 inside-out patches from vesicles that displayed a cGMP-induced  $\Delta[\text{Ca}^{2+}]_i$ . The channels *in situ* exhibited rapid flickering gating and a single-channel conductance of  $< 10 \text{ pS}$  (Fig. 3e, f). Similar cGMP-activated currents could also be recorded from inside-out patches of plasma membrane of human (Fig. 3g) and bovine (data not shown) sperm. Seven of roughly 150 excised membrane patches exhibited cGMP-activated currents between 3 and 12 pA.

The small current amplitudes and the low success rate of detecting CNG channel activity indicate that the channel density is at least two orders of magnitude lower than in rod outer

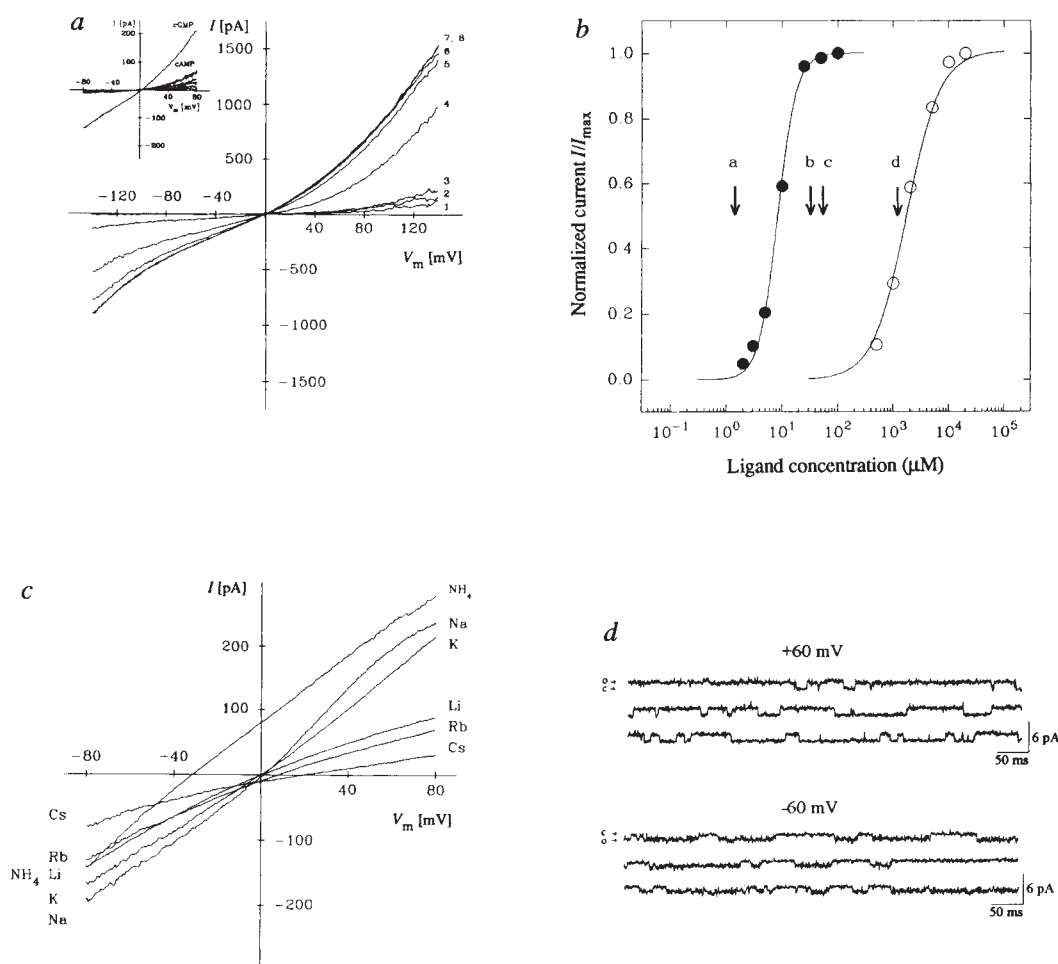
segments or in cilia of olfactory sensory neurons<sup>15-18</sup>.

The tissue-specific expression of channel messenger RNA was analysed by northern blotting. Transcripts of  $\sim 2.8$  kilobases (kb) and  $\sim 4.1$  kb were detected in poly(A)<sup>+</sup> RNA from bovine testis and retina (Fig. 4a). The size of the smaller transcript (2.8 kb) agreed reasonably with the size of the cloned cDNA (2.6 kb). The larger transcript (4.1 kb) may represent an incompletely or alternatively spliced mRNA species. Polymerase chain reaction (PCR) analysis demonstrated that mRNA of the CNG channel species from rod photoreceptors and olfactory epithelium was not expressed in testis tissue, whereas the retina contained both rod and testis CNG channel mRNA (data not shown).

The identity of the testis and retinal CNG channel cDNA was confirmed by isolating a partial clone from a cDNA library of the bovine retina. The nucleotide sequence of the retinal clone was identical to the corresponding sequence of the testis channel cDNA. The missing 5' end of the coding region of the retinal CNG channel was obtained by PCR using retinal cDNA as template and testis channel-specific primers.

Two antibodies, PPc14 and PPc15, were raised against a

FIG. 2 Electrophysiological properties of the testis channel from inside-out patches of *Xenopus* oocytes. a, Current-voltage ( $I$ - $V$ ) recordings in the absence of divalent cations. cGMP concentrations: trace 1,  $2 \mu\text{M}$ ; trace 2,  $3 \mu\text{M}$ ; trace 3,  $5 \mu\text{M}$ ; trace 4,  $10 \mu\text{M}$ ; trace 5,  $25 \mu\text{M}$ ; trace 6,  $50 \mu\text{M}$ ; trace 7,  $100 \mu\text{M}$ ; trace 8,  $1,000 \mu\text{M}$ . Inset,  $I$ - $V$  recordings at a saturating cGMP concentration ( $100 \mu\text{M}$ ) and increasing cAMP concentrations (0.5, 1, 2, 5, 10 and  $20 \text{ mM}$ ). b, Ligand sensitivity determined from normalized currents  $I/I_{\text{max}}$  at  $V_m = +80 \text{ mV}$ . Smooth curves represent a least-squares fit of the Hill equation  $I/I_{\text{max}} = C^n/(C^n + K_{1/2}^n)$  to the experimental data; C, concentration of the ligand. ●, cGMP; ○, cAMP;  $K_{1/2}$  (cGMP) =  $8.3 \pm 2.1 \mu\text{M}$ ; Hill coefficient  $2.6 \pm 0.4$  ( $n=10$ );  $K_{1/2}$  (cAMP) =  $1,720 \pm 456 \mu\text{M}$ ; Hill coefficient  $1.5 \pm 0.2$  ( $n=7$ ). Arrows indicate the  $K_{1/2}$  values of the cloned olfactory channel (a, cGMP; c, cAMP) and the rod channel (b, cGMP; d, cAMP)<sup>25</sup>. c, Ionic selectivity of the testis CNG channel.  $I$ - $V$  recordings under symmetrical bi-ionic conditions. The pipette solution contained 100 mM KCl and the perfusion solution 100 mM of the respective monovalent cation;  $100 \mu\text{M}$  cGMP. The following values for  $V_{\text{rev}}$  (mean  $\pm$  s.d. (number of experiments)) were obtained:  $\text{NH}_4^+$ ,  $-34.3 \pm 3.1 \text{ mV}$  ( $n=10$ );  $\text{Na}^+$ ,  $+1.7 \pm 1.2 \text{ mV}$  ( $n=9$ );  $\text{Li}^+$ ,  $+2.7 \pm 1.6 \text{ mV}$  ( $n=6$ );  $\text{Rb}^+$ ,  $+8.1 \pm 0.5 \text{ mV}$  ( $n=7$ ); and  $\text{Cs}^+$ ,  $+19.3 \pm 1.5 \text{ mV}$  ( $n=3$ ). d, Traces of single-channel currents induced by  $25 \mu\text{M}$  cGMP. METHODS. A recombinant clone, pCGTEF, that contained a perfect



Kozak consensus sequence<sup>26</sup> and the 5' and 3' non-coding regions of a  $\beta$ -globin gene from *Xenopus laevis* (vector pGEMHE) was constructed by PCR techniques. Channel-specific cRNA was expressed in oocytes as previously described<sup>27</sup>. The solution in the pipette and the perfusion medium contained (in mM): 100 KCl, 10 EGTA/KOH, 10 HEPES/KOH, pH 7.2. Macroscopic currents of excised inside-out patches were recorded under voltage-clamp conditions as described<sup>25</sup>. Single-channel currents were digitally filtered at 750 Hz.



fusion protein containing the C-terminal domain of the testis channel polypeptide (amino-acid residues 593–706). PPc15 specifically stained the outer segments of cone photoreceptor cells (Fig. 4b), whereas PPc14 also labelled the outer segments of rod photoreceptors. These results indicate that the same channel polypeptide is expressed in sperm and in cone photoreceptors. Attempts to detect CNG channel-specific immunoreactivity in cryosections from testis tissue or in isolated sperm cells were unsuccessful. We attribute this failure to the extremely low density of the channel polypeptide in sperm membranes.

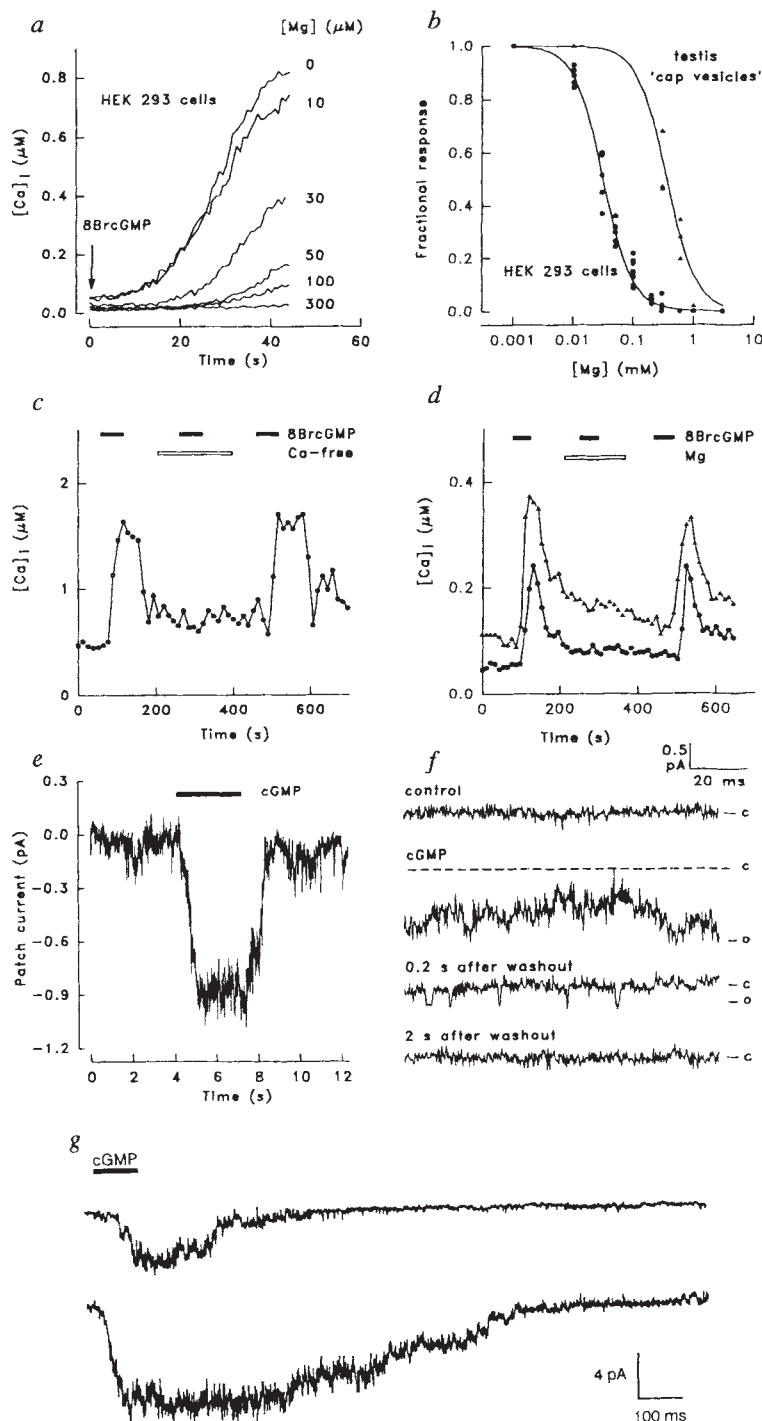
Antibodies PPc14 and PPc15 recognized a major band of  $M_r \sim 82$ K in western blots of membranes from HEK 293 cells

transfected with the testis channel cDNA (Fig. 4c, lane 3). Mock transfected HEK 293 cells did not express the  $\sim 82$ K protein (Fig. 4c, lane 2). The  $M_r$  of the channel polypeptide expressed in HEK 293 cells is virtually identical to the  $M_r$  value expected from the deduced amino-acid sequence. Antibody PPc14, which in western blotting was more sensitive than PPc15, recognized a band of  $\sim 70$ K in sperm membranes (Fig. 4c, lane 4). These results suggest that the CNG channel in sperm may also exist in a proteolytically processed form as has been previously reported for the bovine rod<sup>19</sup> and the chicken rod and cone CNG channels<sup>10</sup>.

In summary, we have identified a novel CNG channel expressed in sperm and in cone photoreceptors. Properties of

**FIG. 3** Identification of functional CNG channels by  $\text{Ca}^{2+}$  imaging and patch-clamp recording. **a**, Changes in  $[\text{Ca}^{2+}]_i$  induced by 1 mM 8Br-cGMP and inhibition by external  $\text{Mg}^{2+}$  in Fura-2/AM-loaded HEK 293 cells, transfected with CGTE cDNA, 1 mM external  $\text{Ca}^{2+}$ . **b**, Dose-response relationship of the  $\text{Mg}^{2+}$  blockage of 8Br-cGMP-induced  $\text{Ca}^{2+}$  flux into HEK 293 cells (circles,  $K_i = 31 \mu\text{M}$ , Hill coefficient = 1.8) and testis 'cap' vesicles (triangles,  $K_i = 300 \mu\text{M}$ , Hill coefficient = 1.8). **c**,  $\Delta[\text{Ca}^{2+}]_i$  elicited by 1 mM 8Br-cGMP in 'cap' vesicles is dependent on extracellular  $\text{Ca}^{2+}$ . **d**, Inhibition of  $\Delta[\text{Ca}^{2+}]_i$  in 'cap' vesicles by 1 mM external  $\text{Mg}^{2+}$ ; 8Br-cGMP 1 mM,  $\text{Ca}^{2+}$  3 mM. **e**, cGMP-activated current in an inside-out patch of a 'cap' vesicle ( $V_m = -50$  mV; 100  $\mu\text{M}$  cGMP). **f**, cGMP-activated currents at higher time resolution. Shortly after washout of cGMP (third trace) single-channel events ( $\sim 6.5$  pS) became visible; 2 s after the washout, the single-channel events disappeared (fourth trace). **g**, cGMP-activated currents in excised inside-out patches of human sperm membranes at 100  $\mu\text{M}$  cGMP (upper trace) and 1 mM cGMP (lower trace);  $V_m = -50$  mV.

**METHODS.** **a**, **b**, For transient expression, the coding region of pCGTE was subcloned into the expression vector pcDNA1 (Invitrogen) and transfected<sup>28</sup> into HEK 293 cells. Cells were loaded with 1  $\mu\text{M}$  Fura-2/AM for 45 min at 37 °C in the presence of 0.02% Pluronic F-127.  $[\text{Ca}^{2+}]_i$  was measured by Fura-2 fluorescence image analysis (Applied Imaging). The test solution contained (in mM): 120 NaCl, 4.5 NaOH, 4.5 KCl, 10 HEPES, 10 glucose, pH 7.4.  $\text{Mg}^{2+}$  contamination was  $\leq 2 \mu\text{M}$  as determined by spectrophotometric analysis. 8Br-cGMP (1 mM) was applied for 30 s (arrow). **c**, **d**, Testis tissue was incubated for 30 min at 37 °C in Krebs-Ringer solution containing glucose, protease inhibitors, DNase I, collagenase and hyaluronidase. The cell suspension was filtered through a cascade of nylon meshes (150, 100, 50, 30  $\mu\text{m}$ ). Cells were collected by centrifugation and washed three times. **e**, **f**, For the patch-clamp experiments, the solution on both sides of the membrane contained (in mM): 120 NaCl, 25 NaOH, 10 EGTA, 10 HEPES, pH 7.4. The records were low-pass filtered at 0.5 kHz. **g**, Ejaculated semen was washed by centrifugation to remove seminal fluid. Sperm were hypo-osmotically swollen<sup>29</sup> and then placed in a poly-L-lysine-treated culture dish. Inside-out patches of plasma membrane were excised mostly from the mid-piece region of sperm cells. Recordings were done in symmetrical solutions containing (in mM): 120 NaCl, 10 EGTA, 1  $\text{CaCl}_2$ , 10 HEPES, pH 7.6, as described<sup>30</sup>.



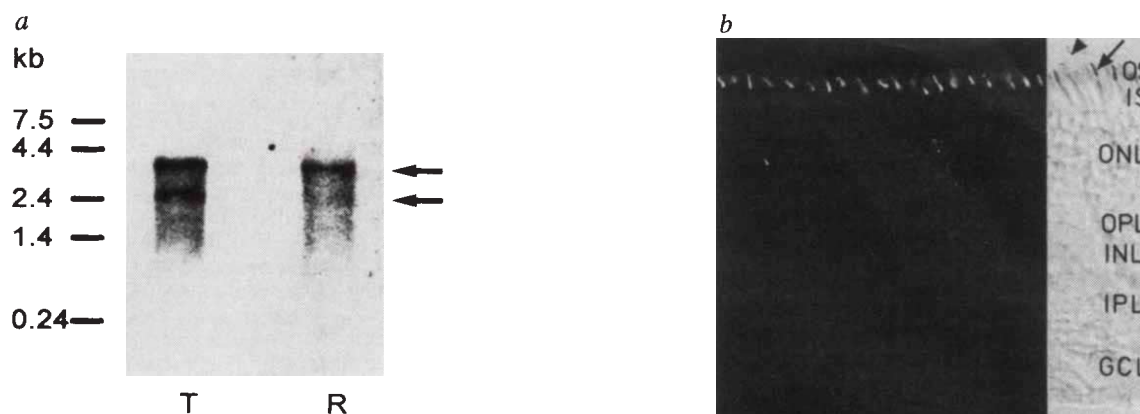


FIG. 4 Site of expression of CNG channel in bovine retina and in testis. **a**, Northern blot analysis of the distribution of CGTE mRNA in poly(A)<sup>+</sup> RNA from bovine testis (T), and retina (R). Two bands of ~2.8 kb and ~4.1 kb from testis (5 h exposure) and retina (50 h) poly(A)<sup>+</sup> RNA hybridized with the testis channel cDNA probe. **b**, Vertical cryosection of a bovine retina stained with antiserum PPc15. Antiserum PPc15 specifically labelled the outer segments of cone photoreceptor cells (arrowhead). Rod outer segments are not stained (arrowhead). Immunoreactivity was visualised with a FITC-conjugated secondary antibody (epi-fluorescence, left side) or with a HRP-conjugated secondary antibody using diaminobenzidine as chromogen (Normarski optics, right side). Scale bar, 50  $\mu$ m. GCL, Ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments. **c**, Western blot analysis of testis CNG channel. Lane 1, membranes of bovine rod outer segments, 5  $\mu$ g protein; lanes 2 and 3, membranes of HEK 293 cells transfected with pcDNA1 vector alone (lane 2) or pcDNA1 vector containing CGTE cDNA (lane 3), 10  $\mu$ g protein each; lanes 4 and 5, bovine sperm membranes, 150  $\mu$ g protein each. Lanes 1–4 were incubated with antibody PPc14 and secondary antibody; lane 5 was incubated only with secondary antibody. PPc14 labelled the rod CNG channel (lane 1), the heterologously expressed testis CNG channel (lane 3) and a 70K protein in sperm (lane 4). The faint upper band seen in lane 3 may represent post-translationally modified channel protein.

**METHODS.** **a**, Poly(A)<sup>+</sup> RNA was isolated using a FastTrack RNA isolation kit (Invitrogen). Northern blot analysis was with 6  $\mu$ g poly(A)<sup>+</sup> RNA. A 2.5-kb restriction fragment from pCGTE was labelled with <sup>32</sup>P by random priming and used as a probe for the northern blots. Hybridization was at

42 °C, 50% formamide; membranes were washed twice with 0.1  $\times$  SSC, 0.1% SDS at 67 °C. **b**, **c**, A cDNA fragment encoding amino-acid residues 593–706 of the C-terminal region of the testis CNG channel was subcloned in pGEX-2T (Promega) expression vector. The purified fusion protein was used to raise polyclonal antibodies PPc14 and PPc15; antibodies were purified from rabbit serum by affinity chromatography. Membrane proteins were separated by SDS-PAGE, transferred to Immobilon membrane and labelled with the polyclonal antibody PPc14. Immunoreactivity was visualized with the ECL detection kit (Amersham). Immunohistochemistry was done as described previously<sup>10</sup>.

the testis CNG channel *in situ* and expressed in *Xenopus* oocytes are similar but not identical. It needs to be established whether the testis and the cone CNG channels *in vivo* exist as heterooligomers as the rod CNG channel<sup>20</sup>, and whether they are proteolytically processed in the same fashion.

Although guanylyl cyclase activity is well documented in invertebrate sperm<sup>9,21</sup>, convincing evidence is lacking for a guanylyl cyclase in vertebrate sperm. Recent studies, however, suggest that vertebrate sperm may use signal transduction pathways

similar to those of photoreceptor and olfactory cells. Members of the gene families of odorant receptors<sup>22</sup>, rhodopsin kinases<sup>23</sup> and arrestin<sup>23</sup> have been identified in vertebrate sperm. It is tempting to speculate that CNG channels are involved in a cGMP-signalling pathway that controls chemotaxis of vertebrate sperm. Future studies will be needed to identify the membrane receptors and ligands that couple to this signalling pathway and to delineate the sequence of events that result in a change in both cGMP and intracellular Ca<sup>2+</sup>. □

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# MHC class I/ $\beta_2$ -microglobulin complexes associate with TAP transporters before peptide binding

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**MAJOR histocompatibility complex class I molecules bind antigenic peptides in the endoplasmic reticulum (ER) and transport them to the cell surface for recognition by cytotoxic T lymphocytes. The peptides are predominantly generated from cytoplasmic proteins, probably by the action of the multicatalytic proteinase complex, or proteasome<sup>1,2</sup>. They are transported into the ER by the transporters associated with antigen processing (TAP), a complex formed from two subunits, TAP.1 and TAP.2 (refs 3–5). Here we show that the TAP molecules are intimately involved in the assembly of the class I/ $\beta_2$ -microglobulin ( $\beta_2$ m) peptide complex. Free class I heavy chains are associated in the ER with the chaperone calnexin<sup>6,7</sup>. In human B-cell lines, however, class I/ $\beta_2$ m dimers in the ER are physically associated with TAP molecules rather than calnexin. Our results suggest that calnexin mediates class I/ $\beta_2$ m dimerization, and subsequent binding of the dimers to TAP molecules facilitates their association with TAP-transported peptides.**

Digitonin extracts of radiolabelled human lymphoblastoid cell lines were immunoprecipitated with a purified rabbit antiserum against the C-terminal peptide of TAP.1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of these precipitates revealed a band of relative molecular mass about 45K (Fig. 1a). This band was not detected when the cells were lysed with the detergent Triton X-100 (Fig. 1a) or with the detergents *N*-octyl glucoside, sodium deoxycholate, or polyoxyethylene dodecylether (data not shown). No 45K band was precipitated from the mutant cell line .221, which lacks all classical HLA class I genes<sup>8</sup> and only a faint band was seen in C1R, a cell line that expresses serologically detectable HLA-Cw4 only<sup>9</sup>. However, anti-TAP.1 precipitates of C1R cells transfected with the HLA-A3 or -B27 genes contained a strong 45K TAP-associated band. These results imply that the band corresponds to major histocompatibility complex (MHC) class I heavy chains. No band was precipitated from the TAP.1/TAP.2-negative cell line T2 (ref. 10), eliminating the possibility of cross-reactivity of the purified rabbit antiserum with class I molecules. TAP.1/TAP.2 bands (70/71K) appear faintly after prolonged exposure and can be seen in the C1R transfectants (Fig. 1) and in Swei cells in Fig. 2. The weak signal may reflect a low turnover rate for TAP molecules relative to the associated newly synthesized class I molecules.

To rule out the possibility that the association occurs after solubilization, unlabelled T0 cells, which contain the TAP.1-associated band (Fig. 1b, lane 1), and radiolabelled T2 cells were mixed before solubilization in digitonin. No association between the labelled class I heavy chain of T2 (Fig. 1b, lane 3) and the unlabelled TAP of T0 was observed, although class I in T2 was well labelled (Fig. 1b, lane 4). To determine whether all or only a limited subset of HLA alleles associate with TAP.1, we analysed the TAP-associated molecules of T0 (ref. 11), which is a T  $\times$  B hybrid expressing all alleles of its fusion partners CEM (A1, B8, Aw30, Bw6) and 721.174, (A2, B5), by two-dimensional polyacrylamide gel electrophoresis (Fig. 1c). Although the positions of all the alleles, with the exception of HLA-A2 (arrows), have not been precisely defined, most of the species precipitated with the monomorphic anti-class I antibody W6/32 were

FIG. 1 Association of class I heavy chains with TAP molecules in digitonin-solubilized human B-LCLs. a, Different human cell lines were lysed in digitonin (D) or Triton X-100 (T) and precipitated with R.RING4C, an affinity-purified rabbit anti-TAP.1 antiserum. In class I-expressing wild-type cells (Swei, Pala) and class I transfectants (C1R.A3, C1R.B27)<sup>18</sup>, a strong band is visible at about 45K, the mobility of HLA class I heavy chains. No such band is visible in a class I negative cell (.221)<sup>8</sup> or a TAP.1/2-negative cell (T2)<sup>10</sup>, and only a weak band is seen in the HLA-A/B-negative cell C1R<sup>9</sup>. The background band appearing in Triton X-100 lysates at ~46K is probably actin; it appears also in control precipitations with a purified antiserum (R.FluHAP) against an influenza haemagglutinin peptide (data not shown). b, TAP–heavy chain association does not occur after digitonin solubilization. Lanes 1 and 2, anti-TAP.1 precipitates from <sup>35</sup>S-methionine-labelled T0 or T2 cells; lane 3, anti-TAP.1 precipitate from a mixture of unlabelled T0 cells and labelled T2 cells; lane 4, anti-class I (3B10.7) precipitate from labelled T2 cells. c, Multiple HLA-alleles expressed in one cell line are associated with TAP. Anti-TAP.1 or W6/32 precipitates from digitonin-lysed <sup>35</sup>S-methionine-labelled T0 cells were subjected to two-dimensional gel analysis. The patterns of spots representing heavy chains of the different alleles expressed in T0 are basically identical except for different labelling intensities. The large arrowheads indicate the position of HLA-A2, the smaller ones indicate  $\beta_2$ m. An unknown species precipitating with R.RING4C from digitonin lysates is marked by small arrows. It is also found in anti-TAP precipitates from Swei cells, appearing at about 48K (Fig. 2).

**METHODS.** Rabbit antisera (R.RING4C and R.FluHAP) were raised against synthetic peptides conjugated to keyhole limpet haemocyanin (KLH). Peptides were derived from the C terminus of TAP.1 (RING4)<sup>19</sup> and an HLA-DR1-restricted influenza virus haemagglutinin epitope<sup>20</sup>, both containing an additional cysteine at their N terminus allowing conjugation to KLH<sup>21</sup>. Antisera were used for immunoprecipitation after purification on an affinity matrix (the relevant peptide coupled to Sepharose beads)<sup>22</sup>. Monoclonal antibodies used were W6/32<sup>23</sup>, a monomorphic conformation and  $\beta_2$ m-dependent anti-class I antibody, and 3B10.7<sup>24</sup>, a conformation-independent monoclonal rat anti-human-class-I-heavy-chain antibody. Immunoprecipitations from radiolabelled cells were performed essentially as previously described<sup>13,25</sup>. Briefly, cells were labelled with <sup>35</sup>S-methionine for 15 min and either lysed in 1% digitonin or 1% Triton X-100 in 0.15 M NaCl, 10 mM Tris, pH 7.4 (TS), containing protease inhibitors. After preclearing overnight, 6  $\mu$ g affinity-purified antibodies were used for immunoprecipitation with protein A coupled to Sepharose CL4B. The precipitates from either 5  $\times$  10<sup>5</sup> cells (a, Swei, Pala, .221, T2; b) or 1  $\times$  10<sup>6</sup> cells (a, C1R and transfectants) were subjected to SDS–PAGE (12.5% acrylamide). The gels were fixed, treated with Enlightening (NEN–Dupont, Boston, MA), dried and exposed to film for 15 h (a, Swei, Pala; b) or 3 days (a, .221, T2, C1R and transfectants). For two-dimensional gel analysis, T0 (2  $\times$  10<sup>6</sup> cells) were labelled for 15 min with <sup>35</sup>S-methionine and lysed in digitonin; aliquots were immunoprecipitated with either 6  $\mu$ g purified anti-TAP.1 antiserum or W6/32 monoclonal antibody, respectively. The precipitated material was subjected to isoelectric focusing in the first dimension and SDS–PAGE in the second, as described in detail elsewhere<sup>18</sup>.

included in the TAP.1-associated pattern. It is also clear in these gels that  $\beta_2$ m is present in the TAP.1 immunoprecipitates, implying that it is class I/ $\beta_2$ m dimers that are associated with TAP molecules. The intensity of the  $\beta_2$ m band in anti-TAP precipitates is significantly lower than in W6/32 precipitates (Figs 1a and 2). This may reflect association of newly synthesized labelled class I heavy chains with a pre-existing pool of unlabelled  $\beta_2$ m. Two additional spots of higher *M<sub>r</sub>* are present in the TAP.1-associated set which are absent from the W6/32 precipitates. The nature of these species is unknown.

TAP molecules are restricted to the ER and *cis*-Golgi<sup>12</sup>. Thus, TAP-associated class I molecules should be similarly localized. Class I molecules reactive with W6/32 were transported from the ER to the medial Golgi apparatus, as measured by the acquisition of resistance of their *N*-linked glycans to endoglycosidase H (endo H), beginning at 15 min and almost completed by 60 min (Fig. 2a). However, TAP-associated class I heavy chains

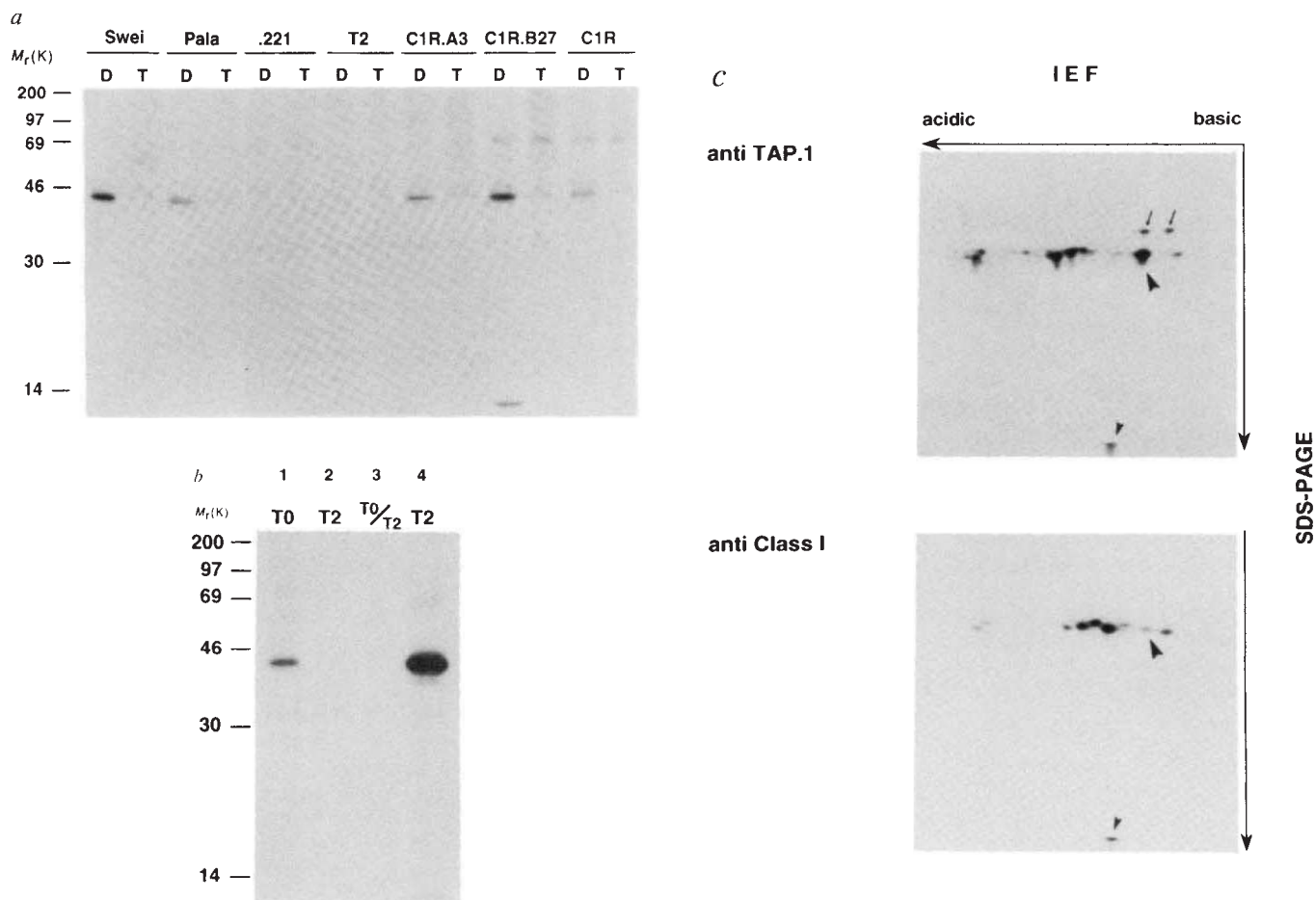
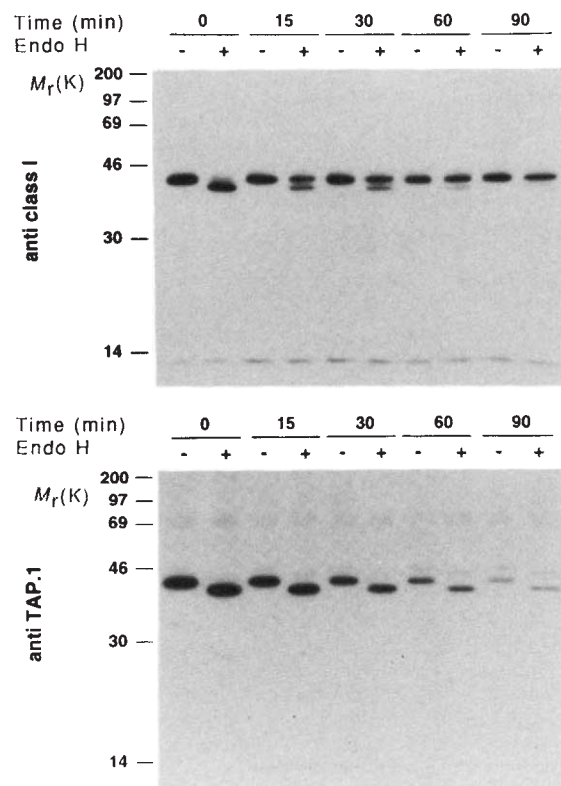


FIG. 2 TAP-associated class I molecules remain in the ER. Free class I molecules, precipitated with W6/32, and TAP-associated heavy chain/ $\beta_2m$  dimers, precipitated with R.RING4C from digitonin lysates of pulse-labelled and chased Swei cells<sup>13</sup>, were digested or mock-digested with Endo H as described<sup>25</sup>. In this exposure (5 days) TAP.1 and TAP.2 bands at about 70 and 71K (marked with arrowheads) are visible. In addition, the coprecipitating molecules seen in Fig. 1c appear after 15 min chase as one band at 48K which is Endo H-sensitive (small arrows). In contrast to the class I dimers, the intensity of this band does not decrease with time, indicating that these molecules remain associated with TAP.

remained endo H-sensitive (Fig. 2b). The intensity of the TAP-associated heavy chain and  $\beta_2m$  bands decreased during the chase, indicating that class I molecules were progressively dissociating from TAP. A band of about 48K, probably corresponding to the two unidentified spots in Fig. 1c, was also apparent, and is glycosylated, on the basis of its susceptibility to endo H.

Mouse class I heavy chains associated with  $\beta_2m$  remain bound to calnexin before peptide binding<sup>7</sup>. However, class I heavy chains from digitonin lysates of the human B-LCL Swei could not be coprecipitated with the anti-calnexin antibody AF8 (Fig. 3), even after a short pulse-labelling of 3 min (data not shown). Calnexin association of heavy chains was clearly seen in the  $\beta_2m$ -negative cell line Daudi (Fig. 3), as previously reported<sup>6,7</sup>. Formally, this could be interpreted to suggest that class I heavy chains that lack associated  $\beta_2m$  are bound to calnexin *en route* to degradation. However, calnexin is generally found to associ-





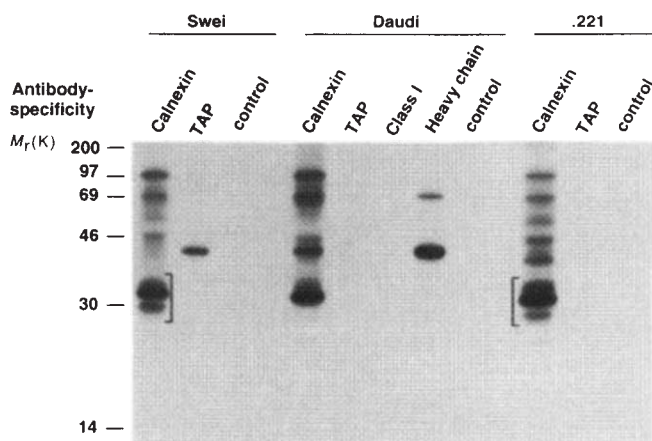


FIG. 3  $\beta_2m$  is a prerequisite for association of heavy chain and TAP. Swei, Daudi and .221 cells were  $^{35}\text{S}$ -methionine labelled for 15 min. Calnexin and associated molecules were precipitated from digitonin-lysed cells with the monoclonal antibody AF8 (ref. 6). A heavy-chain band can be identified only in the  $\beta_2m$ -negative cell Daudi. In all three cell lines shown here, strong bands representing MHC class II-invariant chain complexes are associated with calnexin<sup>13</sup> (indicated by brackets). TAP.1 and associated molecules were precipitated with R.RING4C. A heavy-chain band appears only in the  $\beta_2m$ - and class I-positive cell Swei. R.FluHAP was used as a negative control antibody. 3B10.7 and W6/32, respectively, show the presence of free heavy chains but not class I/ $\beta_2m$  dimers in Daudi cells. The heavy-chain-specific antibody 3B10.7 also precipitates an unidentified species of ~69K from digitonin lysates. Methods are described in the legend to Fig. 1.

ate with free subunits of multimeric complexes (such as T-cell receptor subunits<sup>6</sup> or MHC class II subunits<sup>13</sup>) during the process of assembly. It seems likely, therefore, that in normal human cells class I heavy chains associate transiently with calnexin, and when  $\beta_2m$  association occurs the newly formed class I- $\beta_2m$  dimers bind to TAP molecules. Whether this represents a real difference in mechanism between human and mouse cannot be assessed until similar TAP association experiments are done in the mouse.

Our findings suggested that immature class I/ $\beta_2m$  dimers are associated with TAP molecules, leading to the hypothesis that this might help peptide binding. To determine whether TAP-associated class I molecules can bind added peptides, immunoprecipitated TAP.1/class I complexes from the cell line C1R.A3 were incubated with the HLA-A3-binding peptide, Nef 7B. The

complexes were washed and the HLA-A3 molecules released from TAP by incubation in Triton X-100. The released class I molecules could be specifically reprecipitated with the  $\beta_2m$ -dependent antibody W6/32 only when the HLA-A3-specific peptide had been present (Fig. 4a), arguing that peptide binding and stabilization of the HLA-A3 molecule had occurred while it was associated with TAP. Residual HLA-A3 heavy chains were precipitated with the conformation-independent antibody 3B10.7 when the TAP.1-HLA-A3 complexes had been incubated with either Nef 7B or a control peptide (Fig. 4a).

The preceding data show that class I/ $\beta_2m$  dimers associated with TAP molecules are peptide-free and can be loaded with peptides *in vitro*. Peptide-associated class I molecules are normally transported from the ER to the cell surface, suggesting that peptide binding *in vivo* must induce their dissociation from

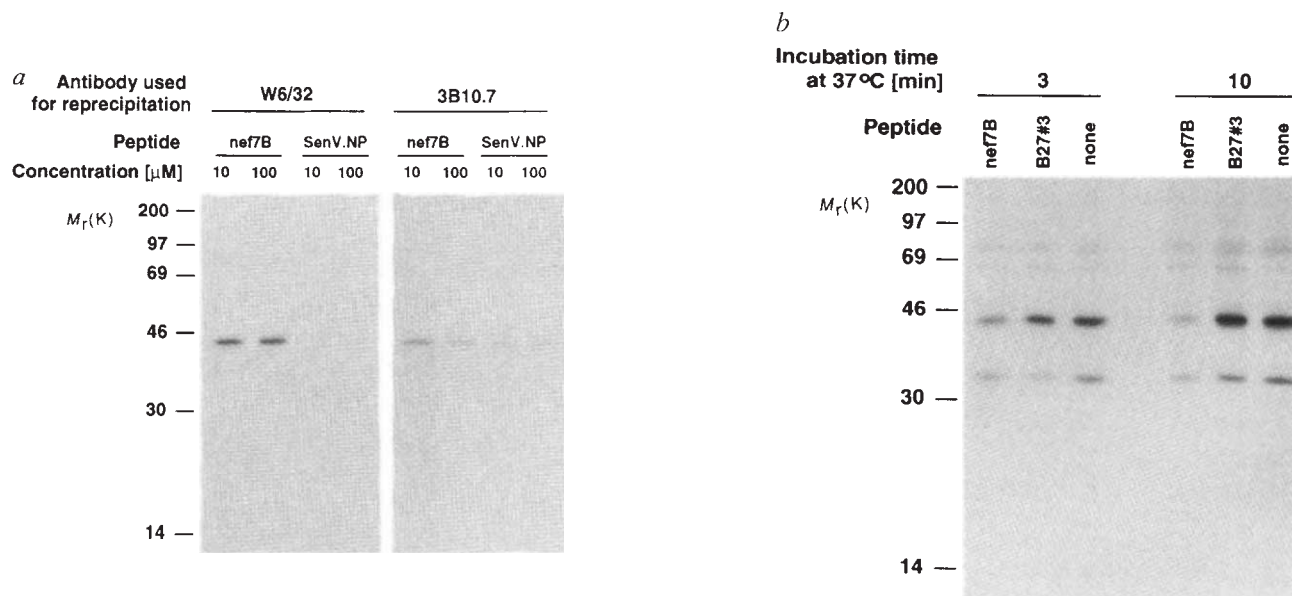


FIG. 4 Peptide binding and TAP association. a, TAP-associated class I molecules are devoid of peptide. Anti-TAP precipitates from pulse-labelled and digitonin-solubilized C1R.A3 cells were washed and incubated in 1% digitonin with different peptide concentrations. Peptides were the HLA-A3-binding Nef 7B peptide<sup>26</sup> and the H-2K<sup>b</sup>-binding Sendai virus nucleoprotein peptide (SenV NP)<sup>27</sup>. After incubation overnight at 4 °C, the precipitates were washed and incubated in 1% Triton X-100 in TS at 37 °C for 1.5 h. Supernatant material was first reprecipitated with W6/32 and subsequently with 3B10.7. b, Peptide binding induces dissociation of class I molecules from TAP *in vivo*. Pulse-labelled

C1R.A3 cells permeabilized with streptolysin O were incubated at 37 °C for 3 or 10 min, with an ATP-generating system, HeLa cell cytosol and 100 μM peptide (Nef 7B), a B27-binding control peptide B27 #3 (RRYQKSTEL<sup>28</sup> in single-letter amino-acid code), or without peptide. Cells were labelled as described for Fig. 1 and subsequently permeabilized with streptolysin O and incubated with the ATP-generating system, HeLa cell cytosol and with or without peptides essentially as described<sup>5</sup>. Solubilization, precipitation with R.RING4C, SDS-PAGE are described in the legend to Fig. 1.

TAP molecules. To investigate this, pulse-labelled C1R.A3 cells were permeabilized with streptolysin O, allowing peptides to be directly introduced into the cytoplasmic compartment. It was previously shown that, in the presence of an ATP-regenerating system and cytosol, specific peptides can induce the transport of HLA-A3 molecules in such cells, as measured by their acquisition of endo H resistance<sup>5</sup>. Here we show that the rate of dissociation of HLA-A3 molecules from TAP is enhanced by the introduction of the Nef 7B peptide into the cells compared to the addition of a control peptide or no peptide (Fig. 4b).

During assembly of class I MHC molecules *in vitro*, either peptide or  $\beta_2m$  can first form a dimer with heavy chains<sup>14</sup>. *In vivo*, however, the data suggest that the calnexin-mediated association of heavy chain and  $\beta_2m$  occurs first, followed by association of the class I/ $\beta_2m$  dimer with TAP molecules. TAP molecules do not play an obligatory chaperone-like role in MHC class I assembly because class I molecules containing signal sequence-derived peptides can be assembled in TAP-negative cell lines<sup>11</sup>, and functional class I complexes containing antigenic epitopes can be generated in such cell lines if the epitope is introduced into the ER through a conventional signal sequence<sup>15</sup>. However, the physical association between TAP and newly synthesized MHC class I molecules presumably helps the assembly of class I complexes containing peptides translocated by TAP molecules.

The precise nature of the HLA class I/TAP association is unknown. TAP.1 is physically bound to TAP.2 molecules, and the class I association could be with TAP.1 directly, indirectly through TAP.2, or it could be mediated by a third molecule, for example the 48K glycoprotein visible in Figs 1c and 2b. The stoichiometry of the complex also remains to be determined. TAP molecules are promiscuous in the peptides they translocate<sup>3-5,16</sup>, whereas class I MHC molecules are highly selective. Thus the majority of peptides translocated by an individual TAP molecule would presumably not be capable of binding to a single associated class I molecule. Multiple TAP molecules may exist in an array in the ER with multiple class I molecules, which would serve to concentrate the class I/ $\beta_2m$  dimers in the vicinity of the total available pool of translocated peptides. Other key elements of the processing machinery, for example trimming enzymes<sup>17</sup>, may similarly be co-localized. □

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## An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis

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THE tyrosine kinase activity of c-Src is stimulated during mitosis by dephosphorylation of its regulatory tyrosine residue<sup>1-3</sup>. This is associated with increased accessibility of its Src homology-2 (SH2) domain for binding a phosphotyrosine-containing peptide<sup>4</sup>. But physiological targets of activated c-Src in mitosis have not yet been identified. Here we report that a 68K protein (p68) becomes tyrosine-phosphorylated and physically associates with Src during mitosis in mouse fibroblasts. p68 independently binds the Src SH2 and SH3 domains *in vitro* and both domains are required for p68 phosphorylation and binding *in vivo*. p68 is closely related to the p62 protein that is associated with the Ras GTPase-activating protein (GAP)<sup>5</sup> and selectively binds, directly or indirectly, polyribonucleotides. Because the Src SH3 domain also binds heterogeneous nuclear ribonucleoprotein K, these results raise the intriguing possibility that c-Src may regulate the processing, trafficking or translation of RNA in a cell-cycle-dependent manner.

NIH3T3 fibroblasts and NIH3T3-derived c-Src overexpressor cells were arrested in mitosis (Fig. 1). To stabilize tyrosine phosphorylation, some cells were briefly (10 min) treated with protein tyrosine phosphatase (PTP) inhibitors before lysis. Mitotic, but not unsynchronized, NIH3T3 cells contained a tyrosine-hyperphosphorylated ~68K protein (p68) (Fig. 1a, lanes 3-6). Mitosis-specific p68 tyrosine phosphorylation was increased in c-Src overexpressor cells (lanes 9-12). In contrast, phosphotyrosine levels of most tyrosine-phosphorylated proteins were either unchanged or decreased in mitosis. To determine whether tyrosine-phosphorylated p68 was physically associated with c-Src in mitotic cells, c-Src was immunoprecipitated from unsynchronized or mitotic overexpressor cells and the immunoprecipitates incubated with [ $\gamma$ -<sup>32</sup>P]ATP (Fig. 1b). A 68K coimmunoprecipitated protein was phosphorylated *in vitro* in immunoprecipitates from mitotic but not unsynchronized cells (lanes 1, 2). Co-immunoprecipitation and *in vitro* phosphorylation of p68 was enhanced by brief pre-treatment of the cells with vanadate (lanes 3, 4) and was greater in immunoprecipitates from mitotic cells expressing a minimally activated Src mutant, c-Src(W148R/Y416F) [c-Src(RF)] or fully-activated c-Src(Y527F) [c-Src(F527)] (lanes 5-10).

Anti-phosphotyrosine immunoblotting demonstrated greatly increased tyrosine phosphorylation of p68 during mitosis in cells transformed by c-Src(F527) or c-Src(RF) (Fig. 1c, lanes 1, 2, 7, 8). However, p68 was not detectably tyrosine-phosphorylated during mitosis in cells transformed by c-Src lacking the SH3 domain (c-Src[dIA]) or part of the SH2 domain (c-Src[dIB]) (lanes 3-6), in spite of elevated levels of cellular protein phosphotyrosine (Fig. 1; our unpublished observations and ref. 6). Tyrosine-phosphorylated proteins, particularly at ~120K, which coprecipitated with Src from unsynchronized c-Src(Y527F) or c-Src(RF) expressor cells (Fig. 1c, lanes 9, 15), were decreased in anti-Src immunoprecipitates from mitotic cells. In contrast, co-immunoprecipitation of tyrosine-phosphorylated p68 with Src was greatly increased in mitosis (lanes 10, 16). Tyrosine-phosphorylated p68 did not co-immunoprecipitate with c-Src(dIA) or c-Src(dIB), although other tyrosine-phosphorylated proteins co-immunoprecipitated with c-Src(dIA) (lanes 11-14). These data indicate a role of the Src SH3 and SH2 domains in mitotic p68 tyrosine phosphorylation and binding to Src.



To determine whether the SH2 and SH3 domains participated directly in the binding of p68 to Src, Src was immunoprecipitated from c-Src(RF) cells (chosen for experimental practicality) with or without competing recombinant glutathione-S-transferase (GST) fusion proteins containing these domains. Without fusion proteins or with GST alone, tyrosine-phosphorylated p68 coprecipitated with Src from mitotic cell lysates (Fig. 2a, lanes 3–6). Although GST-SH3 did not affect p68 recovery, GST-SH3SH2 completely abolished, and GST-SH2 reduced p68 recovery without affecting Src immunoprecipitation (lanes 7–12). The ability of recombinant Src SH2 domain to compete for binding of p68 to Src (together with the deletion-mutant data) suggests that tyrosine-phosphorylated p68 binds the Src SH2 domain *in vivo*. Indeed, tyrosine-phosphorylated p68 was precipitated from mitotic lysates by immobilized GST-SH2 (data not shown). The greater efficiency of GST-SH3SH2, compared to GST-SH2, in competing for p68 binding suggests that the adjacent SH3

domain may influence the affinity of SH2 for p68, as for binding of a phosphopeptide to the Src-SH2 domain<sup>7</sup>.

The potential of p68 to bind directly to the Src SH3 domain was assessed by affinity-precipitation and western blotting. Tyrosine-phosphorylated p68 was precipitated with immobilized GST-SH3 from c-Src(RF) mitotic lysates to a greater extent than from unsynchronized lysates (Fig. 2b, lanes 7, 8). GST-SH3 affinity-precipitates from c-Src(F527) cell lysates (containing several tyrosine-phosphorylated proteins) demonstrated the specificity of precipitation (lanes 10, 12). Tyrosine-phosphorylated p68 was not precipitated by GST alone, an irrelevant GST fusion (GST-Ras) or GST-Crk( $\Delta$ SH2), which contains the two SH3 domains of Crk (lanes 11, 13, 14). p68 in anti-Src immunoprecipitates from mitotic c-Src(RF) cells or GST-SH3 affinity-precipitates from unsynchronized and mitotic c-Src(RF) cells and unsynchronized c-Src(F527) cells was bound directly on western blots by GST-SH3 (lanes 4, 7, 8, 12) but not by GST

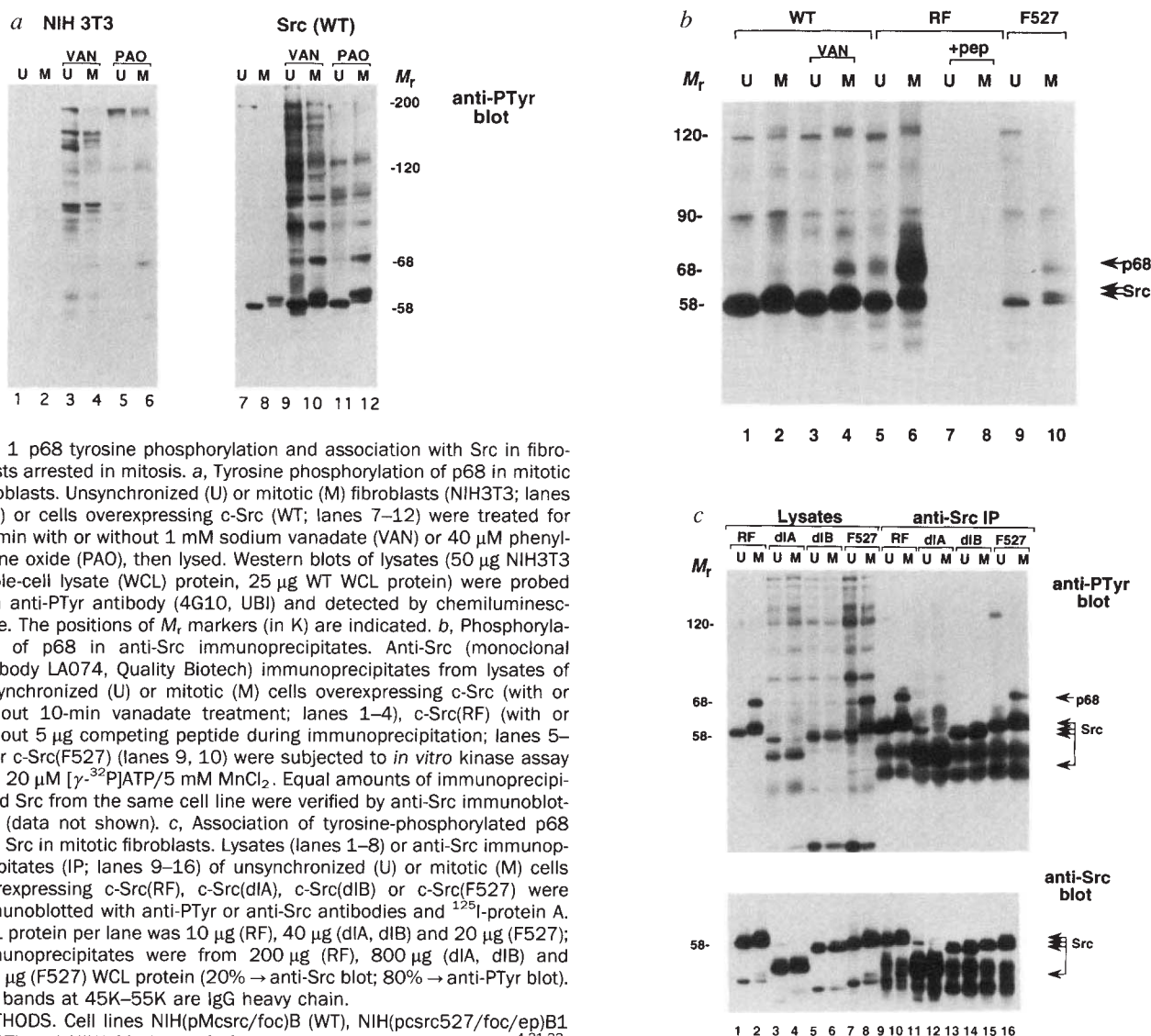


FIG. 1 p68 tyrosine phosphorylation and association with Src in fibroblasts arrested in mitosis. **a**, Tyrosine phosphorylation of p68 in mitotic fibroblasts. Unsynchronized (U) or mitotic (M) fibroblasts (NIH3T3; lanes 1–6) or cells overexpressing c-Src (WT; lanes 7–12) were treated for 10 min with or without 1 mM sodium vanadate (VAN) or 40  $\mu$ M phenylarsine oxide (PAO), then lysed. Western blots of lysates (50  $\mu$ g NIH3T3 whole-cell lysate (WCL) protein, 25  $\mu$ g WT WCL protein) were probed with anti-PTyr antibody (4G10, UBI) and detected by chemiluminescence. The positions of  $M_r$  markers (in K) are indicated. **b**, Phosphorylation of p68 in anti-Src immunoprecipitates. Anti-Src (monoclonal antibody LA074, Quality Biotech) immunoprecipitates from lysates of unsynchronized (U) or mitotic (M) cells overexpressing c-Src (with or without 10-min vanadate treatment; lanes 1–4), c-Src(RF) (with or without 5  $\mu$ M competing peptide during immunoprecipitation; lanes 5–8) or c-Src(F527) (lanes 9, 10) were subjected to *in vitro* kinase assay with 20  $\mu$ M [ $\gamma$ -<sup>32</sup>P]ATP/5 mM MnCl<sub>2</sub>. Equal amounts of immunoprecipitated Src from the same cell line were verified by anti-Src immunoblotting (data not shown). **c**, Association of tyrosine-phosphorylated p68 with Src in mitotic fibroblasts. Lysates (lanes 1–8) or anti-Src immunoprecipitates (IP; lanes 9–16) of unsynchronized (U) or mitotic (M) cells overexpressing c-Src(RF), c-Src(dIA), c-Src(dIB) or c-Src(F527) were immunoblotted with anti-PTyr or anti-Src antibodies and <sup>125</sup>I-protein A. WCL protein per lane was 10  $\mu$ g (RF), 40  $\mu$ g (dIA, dIB) and 20  $\mu$ g (F527); immunoprecipitates were from 200  $\mu$ g (RF), 800  $\mu$ g (dIA, dIB) and 400  $\mu$ g (F527) WCL protein (20%  $\rightarrow$  anti-Src blot; 80%  $\rightarrow$  anti-PTyr blot). The bands at 45K–55K are IgG heavy chain.

**METHODS.** Cell lines NIH(pMcsrc/foc)B (WT), NIH(pcsrc527/foc/ep)B1 (F527) and NIH(pMdlBsrc/pSV2neo/cos)B have been described<sup>4,21,22</sup>. Cell lines NIH(pR148416/pSV2neo/cos)E (RF) and NIH(pMdlAsrc/pSV2neo/cos)A were selected by G418 resistance after cotransfection of plasmids pR148416 and pMBHdIA with pSV2neo. Plasmid pMBHdIA was constructed from pM5HHB5 and pRSVBHdIA (a gift from J. Brugge<sup>6</sup>) as described for pMBHdIB<sup>4</sup>. pR148416, encoding c-Src(W148R/Y416F) was made by replacing a *Sall*–*Pf*MI fragment of pM5H with the corresponding fragment from a p5H-derived plasmid bearing the W148R mutation (gift from H. Hanafusa<sup>23</sup>) and introducing relevant sequences

from pcsrc416 (ref. 22). Mitotic cells were collected after nocodazole treatment<sup>24</sup> and unsynchronized cells were collected by brief trypsinization. Lysates were prepared by washing cells with ice-cold PBS, incubating in 1 ml of PBS at 37 °C for 10 min with or without PTP inhibitors and lysing pellets in 3T3 lysis buffer (1% NP-40, 0.25% sodium deoxycholate, 25 mM HEPES pH 7.5, 150 mM NaCl, 25 mM NaF, 1 mM EDTA, 1 mM sodium vanadate, 10  $\mu$ g ml<sup>-1</sup> leupeptin, 10  $\mu$ g ml<sup>-1</sup> aprotinin).

(lane 12). Similar results were obtained using biotinylated or  $^{35}\text{S}$ -labelled GST and GST-SH3 (not shown).

Purification of tyrosine-phosphorylated p68 from mitotic cells by anti-PTyr affinity chromatography yielded insufficient material. So we purified p68 by SH3 affinity chromatography from unsynchronized c-Src(F527)-transformed cells, in which p68 is basally tyrosine-phosphorylated (Fig. 2b). Proteins were bound to immobilized GST-SH3 and washed stringently to elute non-specific and low-avidity binding proteins. A tyrosine-phosphorylated protein with the same electrophoretic mobility as mitotically tyrosine-phosphorylated p68 was, by Coomassie staining, one of the major proteins recovered. Other predominant purified proteins migrated at  $M_r$ s 65K (p65) and 100K (p100) (data not shown). Proteolytic peptides from these proteins were sequenced. One peptide each from p65 and p100 were 100% identical to human and *Xenopus* heterogeneous nuclear ribonucleoprotein K (hnRNP K)<sup>8,9</sup>, and human, rat and *Drosophila* dynamin<sup>10-12</sup> (Fig. 3a). Dynamin, a microtubule-associated GTPase, has been shown to bind to SH3 domains from a number of proteins, including Src<sup>13</sup>. Two peptides derived from p68 were 100% (7 out of 7) and 95% (18 out of 19, with one conservative change) identical to sequences from the human GAP-associated p62 clone<sup>5</sup> (Fig. 3a). The apparent identity of p68 and GAP-associated p62 was confirmed using antibodies against recombinant p62 (residues 103–281) which detected a 68K protein that co-immunoprecipitated with mitotic Src (Fig. 3b, lane 14), was affinity-precipitated by GST-SH3 (lanes 17, 18) and had identical electrophoretic mobility to p68 detected with anti-PTyr antibody (lanes 4, 8). (Immunoprecipitating antibodies were not

available for the converse experiment.) Although the sequencing and immunological data indicate that p68 is the mouse homologue of the human GAP-associated p62 clone, we have not detected p68 in anti-GAP immunoprecipitates by immunoblotting with anti-PTyr or anti-p62 antibodies. (However, we have co-immunoprecipitated a tyrosine-phosphorylated 62K–64K protein with GAP.) It is possible that p68 represents a post-translationally modified, or alternatively spliced version of p62 which is more reactive with the anti-p62 antibody. Notably, GAP-associated p62 and hnRNP K contain proline-rich sequences which may mediate SH3 binding<sup>5,8</sup> and the p62 C terminus contains several potential tyrosine-phosphorylation and SH2-binding sites<sup>5</sup>.

GAP-associated p62 has homology with RNA-binding proteins and the recombinant protein binds to total cellular RNA on western blots<sup>5</sup>. To examine the RNA-binding potential of p68, lysates from NIH3T3 cells were probed with agarose-immobilized poly(U)<sup>+</sup> in the presence or absence of soluble poly-ribonucleotides (heparin was included to block nonspecific RNA binding). p68, detected with anti-GAP-associated p62 antibody, was efficiently (>50% recovery) precipitated by the poly(U)<sup>+</sup> beads (Fig. 4, lane 2). Soluble poly(U)<sup>+</sup> and, to a lesser extent poly(A)<sup>+</sup>, competed for p68 binding (lanes 5, 7). However, poly(C)<sup>+</sup> or poly(G)<sup>+</sup> were ineffective competitors (lanes 8–11), indicating that p68 binds selectively to poly(U)<sup>+</sup>. This binding might be direct, or indirect via poly(A)<sup>+</sup>-messenger RNA or other proteins. In either case, specific RNA association is suggested. Probing of duplicate western blots with GST or GST-SH3 (with detection by anti-GST antibodies) demonstrated the

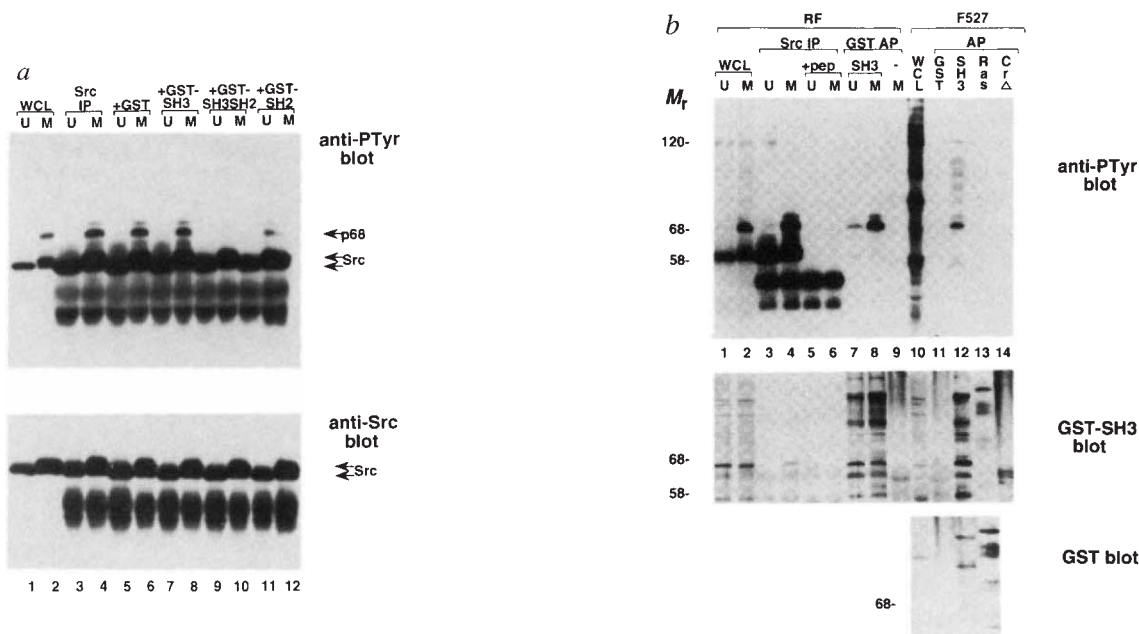


FIG. 2 p68 binding to Src involves the Src SH2 and SH3 domains. **a**, Competition between Src and recombinant Src domains for p68 binding. c-Src(RF) was immunoprecipitated from lysates (250  $\mu\text{g}$  WCL protein) from unsynchronized (U) or mitotic (M) overexpressor cells with anti-Src antibody in the presence of 10  $\mu\text{g}$  of either GST, GST-SH3, GST-SH3SH2 or GST-SH2, or with an equal volume of GST elution buffer containing 5 mM glutathione. Immunoblots of either whole-cell lysates (10  $\mu\text{g}$  WCL protein; lanes 1, 2) or the immunoprecipitates (lanes 3–12) were probed with anti-PTyr (80% of IP) or anti-Src (20%) antibodies. **b**, Direct-binding of p68 to the Src SH3 domain *in vitro*. Unsynchronized (U) or mitotic (M) c-Src(RF) cell lysates were immunoprecipitated with anti-Src (with or without 5  $\mu\text{g}$  antigenic peptide) (IP; lanes 3–6) or affinity-precipitated with GST or GST-SH3 beads (lanes 7–9). Unsynchronized c-Src (F527) cells were affinity-precipitated with GST, GST-SH3, GST-Ras or GST-Crk(ASH2) (lanes 11–14). Precipitates and the whole-cell

lysates (WCL; lanes 1, 2, 10) were western blotted and the blots were probed with anti-PTyr, GST-SH3 or GST alone.

**METHODS.** Generation of pGEX-derived vectors encoding GST-SH3 and GST-SH3SH2 fusion proteins have been described<sup>25</sup>. *Escherichia coli* expressing GST-SH2(Src) were a gift from K. Bibbins<sup>7</sup>, GST-Crk(ASH2) was a gift from B. Knudsen and GST-Ras was a gift from Y. Zheng. Affinity-purified fusion protein concentrations were determined by protein assay (Bio-Rad DC) and verified by SDS-PAGE with Coomassie staining. To probe western blots with fusion proteins, blots were blocked in TTBS (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 2.5% (w/v) BSA and then probed with 2  $\mu\text{g ml}^{-1}$  fusion protein in TTBS containing 1% BSA. Filter-bound fusion proteins were detected by incubation with anti-GST antibodies (Santa Cruz Biotechnology), followed by peroxidase-coupled goat anti-mouse IgG and chemiluminescence.



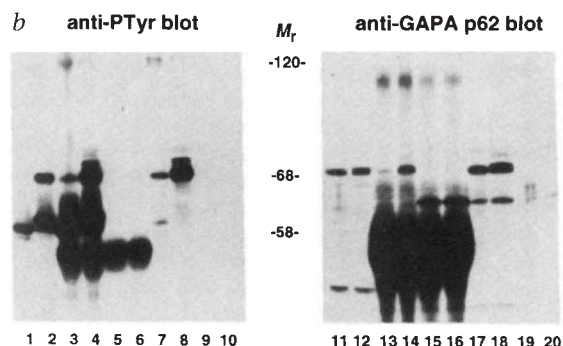
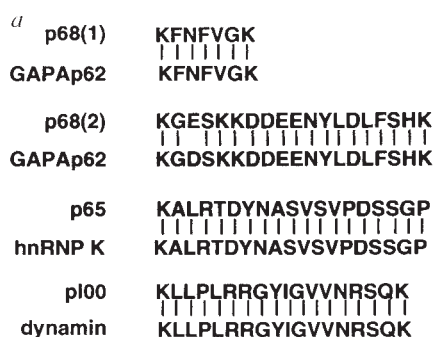


FIG. 3 p68 is closely related or identical to GAP-associated p62. **a**, Sequence alignments of peptides derived from SH3 domain-binding proteins purified from Src-transformed fibroblasts with known protein sequences<sup>9-12</sup>. **b**, Src-associated p68 is recognized by anti-GAP-associated p62 antibodies. Lysates of unsynchronized (odd-numbered lanes) or mitotic (even-numbered lanes) c-Src(RF) overexpressor cells were immunoprecipitated with anti-Src antibody (lanes 3, 4, 13, 14) or affinity-precipitated with GST-SH3 beads (lanes 7, 8, 17, 18). As controls, lysates were incubated with anti-Src in the presence of antigenic peptide (5 µg), protein A-Sepharose beads and GST beads (lanes 5, 6, 15, 16); alternatively, GST or GST-SH3 beads were incubated with lysis buffer alone to identify crossreacting, fusion protein-derived proteins (lanes 9, 10, 19, 20). Blots (from the same gel) were probed with either anti-PTyr (10 µg WCL protein; 200 µg IP; 100 µg AP) or anti-GAP-associated (GAPAp62 (Santa Cruz Biotechnology) (30 µg WCL; 600 µg IP; 300 µg AP) and detected by chemiluminescence.

**METHODS.** Src SH3 domain-binding proteins were purified from NIH 3T3 cells transformed by c-Src(Y527F) by affinity chromatography on GST-SH3 beads. Cells were grown to high density, collected by trypsinization, washed once with PBS, frozen in liquid N<sub>2</sub> and stored at -70 °C until use. Frozen cells from 64 plates (10 cm) were thawed into 24 ml 3T3 lysis buffer containing 0.25% SDS, lysed for 20 min and the lysate was cleared by centrifugation. Cell lysate (150 mg protein, ~25 ml) was pre-cleared with 100 µl of GST adsorbed to glutathione-Sepharose

beads (GST beads) by incubation with rocking for 45 min, followed by centrifugation. Pre-cleared lysate (100 mg protein, 20 ml) was incubated with 35 µl of GST-SH3 beads for 90 min. As controls for specificity and to identify proteins from the fusion protein, pre-cleared lysate (25 mg protein) was incubated with 35 µl of GST beads, or lysis buffer was incubated with 35 µl of GST-SH3 beads. Beads were washed 6 times with 3T3 lysis buffer plus 0.25% SDS, 3 times with wash buffer (0.1% NP-40, 20 mM HEPES (pH 7.5), 1 mM EDTA, 0.1 mM sodium vanadate, 10 µg ml<sup>-1</sup> leupeptin, 10 µg ml<sup>-1</sup> aprotinin) containing 1 M NaCl, 3 times with wash buffer, once with wash buffer containing 5 mg ml<sup>-1</sup> polyproline, and finally 3 times with wash buffer. The low salt concentration in wash buffer was pre-determined to elute many proteins, presumably binding by low-affinity nonspecific hydrophobic interactions with SH3. Fusion proteins and bound proteins were eluted with SDS-PAGE sample buffer. To check the yield and specificity of binding of recovered proteins, proteins (from 20 mg lysate) eluted from GST and GST-SH3 beads were subjected to SDS-PAGE (9% acrylamide) and Coomassie staining. For preparation for microsequencing, the remaining GST-SH3 eluates were run on SDS-PAGE, transferred to PVDF (Bio-Rad), briefly stained with Ponceau S, destained, and the relevant stained bands were excised. Sequencing of HPLC-separated peaks after digestion of PVDF-bound proteins with Lys-C protease was done at the Harvard Microchemistry Facility. Standard protein sequence databases were searched for identities or similarities using the BLAST program<sup>26</sup>.

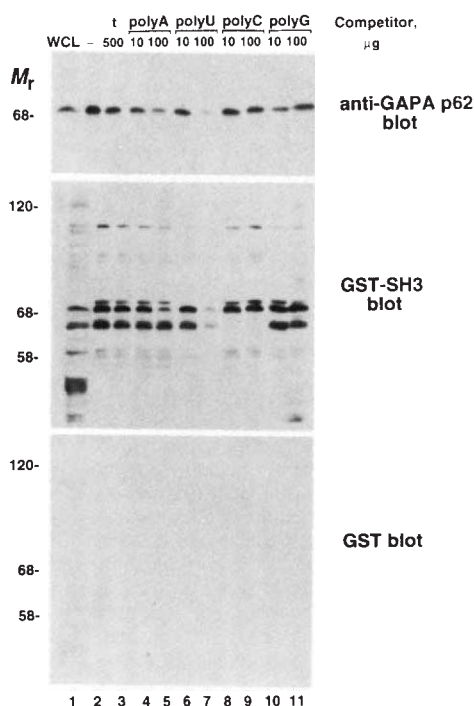


FIG. 4 p68 binds selectively to ribonucleotide homopolymers. NIH3T3 cell lysate was adjusted to 2 mg ml<sup>-1</sup> heparin, and aliquots (~0.4 mg WCL protein) were incubated for 15 min with poly(U)<sup>+</sup>-agarose beads (40-50 µg poly(U)<sup>+</sup>; AGPOLY(U), Type 6, Pharmacia) in the presence or absence (lane 2) of 500 µg of *E. coli* transfer RNA (Boehringer Mannheim) (lane 3) or with 10 µg or 100 µg of the indicated soluble polyribonucleotides (Pharmacia) (lanes 4-11). Washed beads were eluted with SDS-PAGE sample buffer and 25% each of the eluted proteins were probed with anti-p62 antibody, or GST, or GST-SH3 (2 µg ml<sup>-1</sup>), followed by anti-GST antibodies. Unprocessed WCL protein (~25 µg) was directly blotted in lane 1. Bound antibodies were detected with goat anti-mouse IgG-peroxidase and chemiluminescence. *M<sub>r</sub>* markers (in K) are indicated. Concentrations of polyribonucleotides were determined from the absorbance at 260 nm.

presence of three major poly(U)<sup>+</sup>- and GST-SH3-binding proteins of apparent *M<sub>r</sub>* ~65K, ~68K and ~69K (lane 2). The polyribonucleotide binding profile of the 68K SH3-binding protein was the same as that of p68, suggesting their co-identity. Binding of the ~65K SH3-binding protein to poly(U)<sup>+</sup> beads was completely abolished by soluble poly(C)<sup>+</sup> (lanes 8, 9), suggesting its identity with hnRNP K (Fig. 3a), which has a strong binding preference for poly(C)<sup>+</sup> (ref. 8).

Interaction of p68 with Src involves the SH2 and SH3 domains of Src. The SH2 domain presumably binds phosphotyrosine-containing sequences within p68, whereas the SH3 domain may provide an additional p68-binding site or may mediate transient p68-Src binding before tyrosine phosphorylation and SH2 binding. Because the p85 subunit of phosphoinositide 3-kinase and the Src substrate AFAP-110 can also bind both the SH3 and SH2 domains of Src<sup>14,15</sup>, this may represent a general mechanism of effector recognition by Src. The presence of numerous potential tyrosine-phosphorylation sites in the C term-

inus of GAP-associated p62 (ref. 5) suggests that their phosphorylation might recruit other SH2-containing proteins to the Src-p68 complex to initiate mitotic signalling pathways. The p62 sequence also contains a KH domain<sup>9,16</sup>, which has been found in a number of RNA-binding proteins, including hnRNP K<sup>9</sup>, yeast MER1 (involved in meiosis-specific mRNA splicing)<sup>17</sup> and FMR1 (product of the fragile X gene)<sup>18,19</sup>. Our findings that p68 can specifically bind, directly or indirectly, to poly(U)<sup>+</sup> and that hnRNP K binds the Src SH3 domain, suggest a link between Src, SH3 domains and the regulation of RNA processing. Although the Src-p68 interaction is apparent in mitotic cells, its effects could be exerted elsewhere in the cell cycle. For instance, G1 protein expression could be controlled by stabilization or localization of specific RNAs in mitosis. p68 could also play a role in the disassembly of hnRNP complexes at the end of mitosis<sup>20</sup> or it might be programmed by its mitotic phosphorylation to participate in nuclear RNA processing after mitosis. □

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## A target for Src in mitosis

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THE activity of the c-Src protein is increased during cell-cycle stage G1 in fibroblasts stimulated with certain growth factors<sup>1-4</sup>, and at the G2/M transition<sup>5</sup>, but little is known about Src substrates in these circumstances. In contrast, cells transformed with activated Src contain many tyrosine-phosphorylated proteins. We compared the phosphotyrosine content of growing and mitotically arrested Src-transformed cells. We report here that although phosphorylation of most proteins was unchanged during mitosis, phosphorylation of one of about 68K was greatly enhanced. The p68 was physically associated with activated c-Src, and it bound to the SH3 domain of c-Src *in vitro*. Tyrosine-phosphorylated p68 was also present in mitotic extracts of normal cells, suggesting that its phosphorylation was not just a consequence of transformation. Purification and microsequencing of p68 showed that it was related to the previously described GAP-associated protein p62 (ref. 6).

For most of the analyses reported here we used mouse NIH-3T3 cells transformed with an activated allele of c-Src in which the regulatory tyrosine is replaced with phenylalanine (c-Src<sup>Y527F</sup>). Lysates of growing and mitotic cells (arrested with

nocodazole) were probed with antibodies specific for phosphotyrosine (Fig. 1a). Several proteins were identified by this procedure (and have been previously described<sup>7-10</sup>), and the phosphorylation of most of them was either unchanged or reduced in mitosis. However, one protein of relative molecular mass 68K was detected strongly in mitotically arrested cells, but only weakly, if at all, in asynchronously growing cells, and was therefore a good candidate for a mitotic substrate of Src. We also detected tyrosine phosphorylation of p68 in c-Src<sup>Y527F</sup> mitotic cells obtained without nocodazole treatment, indicating that its detection was not an artefact of the drug treatment (data not shown). To determine whether p68 was physically associated with, and a substrate of, c-Src<sup>Y527F</sup>, we did kinase assays on immunocomplexes formed with extracts of growing and mitotic cells and an antibody against the amino terminus of c-Src (anti-2-17). We detected a 68K protein that was associated with and phosphorylated by c-Src derived from mitotic, but not from asynchronous extracts (Fig. 1b), which was also revealed by anti-phosphotyrosine immunoblotting of similar immunocomplexes (Fig. 1b). The detection of p68 was not due to antibody crossreaction because we achieved the same results using an antiserum raised against the C terminus of c-Src (anti-cst. 1). However, associated p68 was only poorly detected when the immunocomplexes were prepared with a third Src-specific antibody, 327 (Fig. 1c). Because the 327 antibody recognizes an epitope at the beginning of the SH3 domain of Src<sup>11</sup>, we speculated that the association of p68 with c-Src<sup>Y527F</sup> might involve the SH3 domain.

We tested whether p68 could associate *in vitro* with SH3 domains (produced as glutathione S-transferase (GST) fusion proteins), using the highly related SH3 domains of the Src family kinases Src and Fyn (Fig. 1d, lanes 1-7). A 68K tyrosine-

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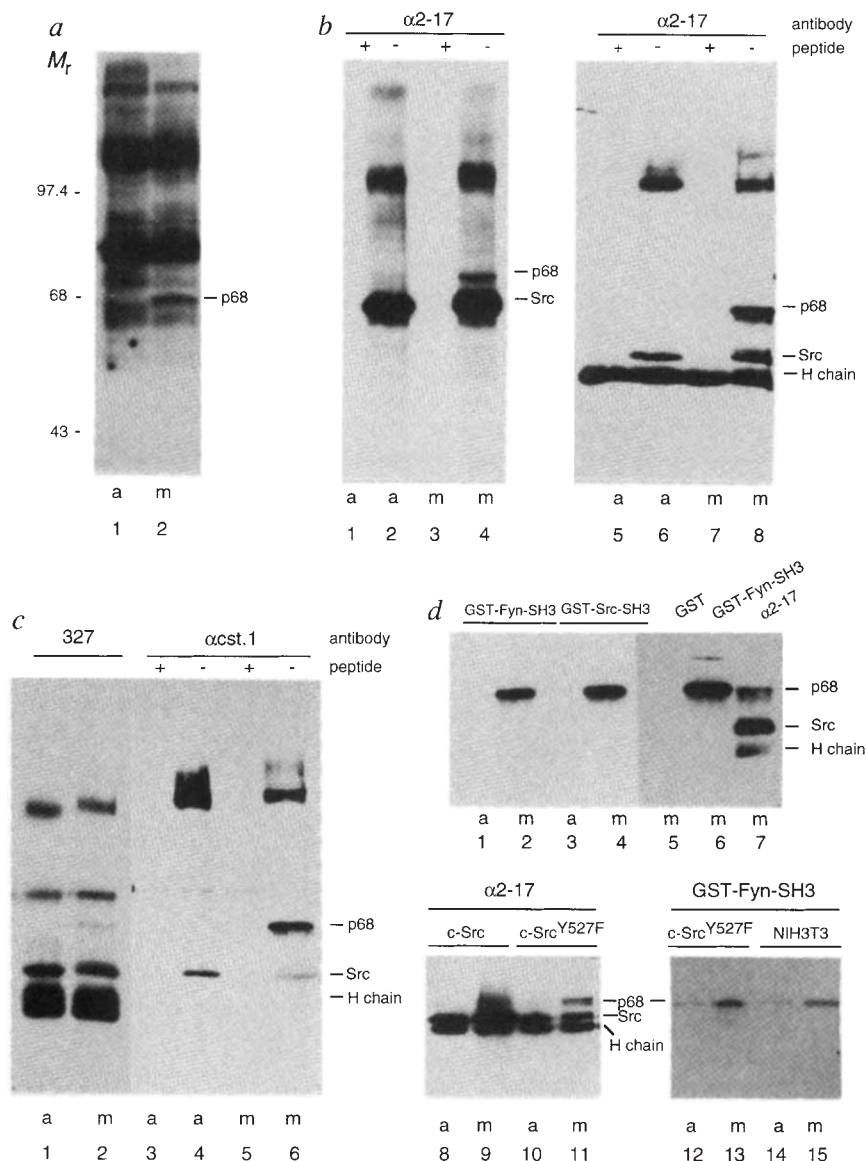
phosphorylated protein, that comigrated with the Src-associated p68, was detected when GST-SH3 constructs were incubated with mitotic, but not interphase, extracts. No binding of p68 to GST alone was detected. We also compared asynchronous and mitotic extracts of fibroblasts overexpressing wild-type c-Src and c-Src<sup>Y527F</sup>, and found that p68 was associated with the mitotic form of Src in both cases (Fig. 1*d*, lanes 8–11). Finally, a 68K tyrosine-phosphorylated, SH3 domain-binding protein was also present in mitotic extracts of normal fibroblasts (Fig. 1*d*, lanes 12–15).

To test whether p68 was present but not tyrosine-phosphorylated in interphase cells, we labelled cells with [<sup>35</sup>S]methionine and purified SH3 binding proteins. A comparison of interphase and mitotic extracts showed few differences, except a slight mobility retardation in a protein of about 68K, which may have been due to phosphorylation (Fig. 2*a*). Anti-phosphotyrosine immunoprecipitation of the same extracts detected a 68K protein from mitotic but not interphase cells (Fig. 2*b*). Protease treatment showed that the 68K proteins isolated from both growing

and mitotic cells by SH3 domain binding and by anti-phosphotyrosine immunoprecipitation were indistinguishable (Fig. 2*c*). We conclude that p68 was present in interphase cells, and able to bind the SH3 domain, but only became tyrosine-phosphorylated during mitosis.

We purified p68 from c-Src<sup>Y527F</sup> cells and obtained the sequence of several peptides (Fig. 3), which showed homology to a known protein, p62 (ref. 12). This protein was first described as becoming tyrosine-phosphorylated in Src-transformed cells and after growth factor stimulation of quiescent fibroblasts<sup>6</sup>. These conditions also lead to association of p62 with the SH2 domain of the GTPase-activating protein of Ras (GAP)<sup>13</sup>. To determine whether p68 was indeed related to p62, we first analysed the c-Src-associated protein by immunoblotting with p62-specific antibodies (Fig. 4*a*). Immunocomplexes made from both growing and mitotic extracts using anti-p62 antibodies contained a doublet at 62K, and a less intense band at 68K (these bands appear faint in the asynchronous extracts shown here, but were clearly visible in other experiments). In c-Src immunoprecipit-

FIG. 1 A 68K protein phosphorylated on tyrosine in mitotic cells, and associated with c-Src. *a*, Anti-phosphotyrosine immunoblot of 25 µg of growing (lane 1: a, asynchronous) and mitotic (lane 2: m, mitotic) c-Src<sup>Y527F</sup> cells. *M<sub>r</sub>* markers (in K) are shown on the left, p68 on the right. *b*, c-Src<sup>Y527F</sup> immunoprecipitated from asynchronous (lanes 1, 2, 5 and 6) and mitotic (lanes 3, 4, 7 and 8) cells, with the Src-specific monoclonal antibody 2-17 (Microbiological Associates) in the presence or absence of competing peptide as indicated, was analysed by kinase assay (lanes 1–4) or anti-phosphotyrosine immunoblotting (lanes 5–8). *c*, c-Src<sup>Y527F</sup> immunoprecipitated from asynchronous (lanes 1, 3 and 4) and mitotic (lanes 2, 5 and 6) cells, with the Src-specific monoclonal antibody 327 (gift from J. Brugge; lanes 1 and 2) or cst.1 (which recognizes Src, Fyn and Yes, but in these overexpressing cells sees predominantly c-Src<sup>Y527F</sup> (ref. 3); lanes 3–6) was analysed by anti-phosphotyrosine immunoblotting. *d*, Top: asynchronous (lanes 1 and 3) and mitotic c-Src<sup>Y527F</sup> extracts (lanes 2, 4–7) were reacted with GST-Fyn-SH3 (lanes 1, 2 and 6), GST-Src-SH3 (lanes 3 and 4), GST (lane 5) or anti-2-17 (lane 7), and analysed by anti-phosphotyrosine immunoblotting. Lanes 1–4 and 5–7 represent independent experiments. Bottom: extracts of asynchronous (lanes 8, 10, 12 and 14) and mitotic (lanes 9, 11, 13 and 15) NIH3T3 cells (lanes 14 and 15) or NIH3T3 cells overexpressing c-Src (lanes 8 and 9) or c-Src<sup>Y527F</sup> (lanes 10–13) were reacted with anti-2-17 (lanes 8–11), or GST-Fyn-SH3 (lanes 12–15), and analysed by anti-phosphotyrosine immunoblotting. METHODS. NIH3T3 cells, and those overexpressing c-Src, or an activated allele, Src<sup>Y527F</sup>, were used. Mitotic block was by 12–15 h incubation with 40 ng ml<sup>-1</sup> nocodazole. Lysis, immunoprecipitations and kinase assays (250 µg lysate) were as described<sup>3</sup> except that RIPA buffer was used throughout. For GST experiments, 100 µg extract (except for the NIH3T3 cells, when 500 µg extract was used) precleared with 10 µg GST coupled to glutathione-Sepharose beads (Pharmacia) were incubated with 10 µg GST, GST-Src-SH3 or GST-Fyn-SH3 immobilized on glutathione-Sepharose beads. After washing with 20 mM HEPES, pH 7.2, 150 mM NaCl, 1% NP-40, 0.3% SDS, proteins were resolved on 8.25% SDS-polyacrylamide gels. Blots were incubated with anti-phosphotyrosine antibodies (UBI; 1:1,500 dilution) and detected



with [<sup>125</sup>I]-labelled antibodies (total extracts), or horseradish peroxidase coupled antibodies followed by chemiluminescence (immunoprecipitates).

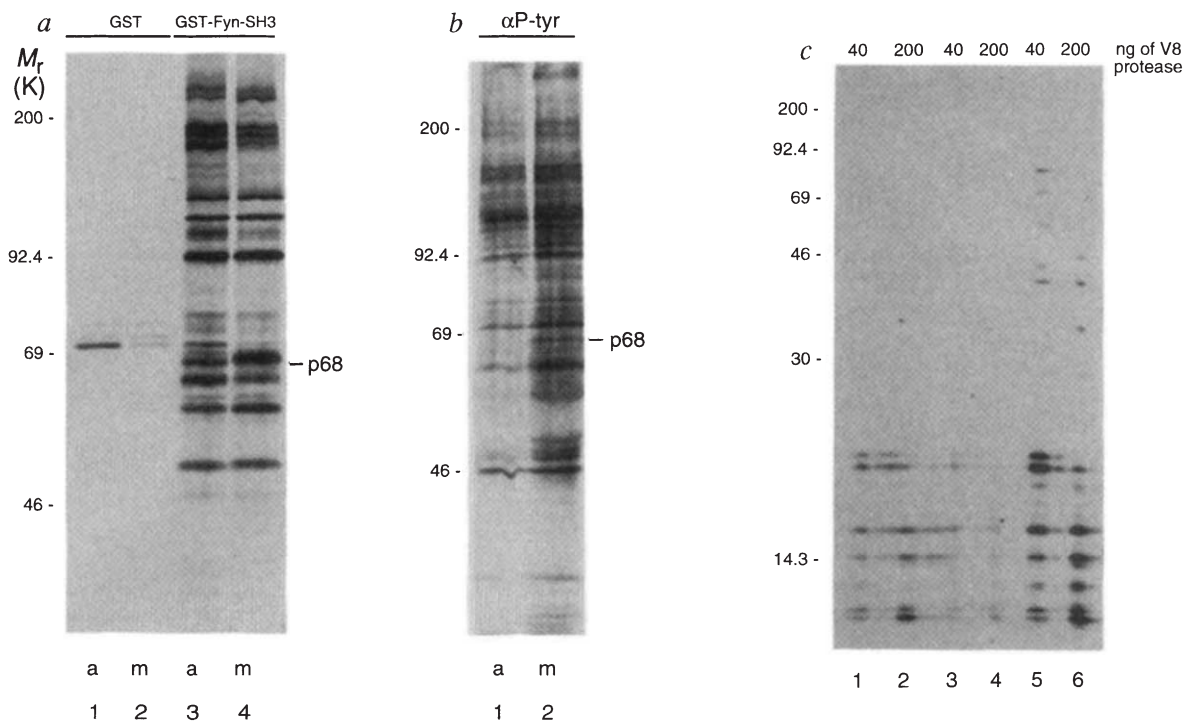


FIG. 2 Detection of p68 in interphase and mitotic extracts. **a**, [ $^{35}$ S]methionine-labelled c-Src<sup>Y527F</sup> growing (lanes 1 and 3) and mitotic (lanes 2 and 4) cell lysates were incubated with either GST (lanes 1 and 2) or GST-Fyn-SH3 (lanes 3 and 4), washed and analysed by SDS-PAGE. **b**, [ $^{35}$ S]methionine-labelled c-Src<sup>Y527F</sup> growing (lane 1) and mitotic (lane 2) cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and analysed by SDS-PAGE. **c**, The p68 bands from gels such as those shown in **a** and **b** were analysed by digestion with 40 and 200 ng *Staphylococcus aureus* V8 protease, and

resolved in 14% polyacrylamide gels. Lanes 1 and 2: the 68K SH3 domain-associated protein from asynchronous extracts; lanes 3 and 4: the 68K protein detected in anti-phosphotyrosine immunoprecipitates of mitotic extracts; lanes 5 and 6: the 68K SH3 domain-associated protein from mitotic extracts.

**METHODS.** Interphase and mitotic extract preparation, immunoprecipitations and GST binding were all as described in the legend to Fig. 1. One-dimensional peptide analysis has been described<sup>22</sup>.

ates, only p68 was detected, and only in the immunoprecipitate of mitotic extracts. We also compared anti-phosphotyrosine immunoblots of anti-p62 immunoprecipitates and SH3 domain-bound proteins (Fig. 4b). Anti-p62 immunoprecipitates contained phosphorylated p62 (present as a doublet) and an associated protein of 120K (probably GAP) in asynchronous extracts.

However, in mitotic extracts a 68K protein that exactly comigrated with SH3-bound p68 was also present. Both the [ $^{35}$ S]methionine-labelled SH3-bound p68 and the p68 labelled in kinase assays were also immunoprecipitable with p62-specific antibodies (data not shown). From all these data, we conclude that p68 is antigenically related to p62. However, note that p62

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1  MQRDDPAAR MSRSSGRSGS MDPGSAHPVS RQTPSRQPPL PHRSRGGGGG
      ASPA TQPPPLLPPS t
51  SRGGARASPA TQPPPLLPPS ATGPDATVGG PAPTPLLPPS ATASVKMEPE
      YLPPELMAE * KDSLDPSFTH AMQLLSVEIE K KD DEENYLhLFS
101 NKYLPPELMAE KDSLDPSFTH AMQLLTAEIE KIQKGDSSKKD DEENYLDLFS
      HK ERV LIPVK
151 HKNMKLKERV LIPVKQYPKF NFGVKILGPO GNTIKRLQEE TGAKISVLGK
      HLNlaengl
201 GSMRDKAKEE ELRKGDDPKY AHLNMDLHVF IEVFGPPCEA YALMAHAMEE
      YLNGVPEP SR
251 VKKFLVPDMM ODICQEQFLE LSYLNGVPEP SRGRGVPVRG RGAAPPPPPV
      PRGRGVGPFR GALVRGTPVR GAITRGATIV RGVPPPTIVR GAPAPRARTA
301
351 GIORIPLPPP PAPETEEYEG YDDTYAEQSY EGYEGYSSQS QGDSEYYDYG
      GAYREHPY GRY
401 HGEVQDSYEA YGDDWNGTR PSLKAPPARP VKGAYREHPY GRY

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FIG. 3 Peptide sequence of p68 and its relation to p62. The predicted amino-acid sequence of the human GAP-associated protein p62 is shown<sup>12</sup>. Peptide sequences obtained from trypsin and lysendopeptidase digests are indicated above the appropriate sequence of p62. The serine residue marked with an asterisk in one peptide indicates a potentially phosphorylated residue. Lower-case letters in the peptide sequence are used to indicate mismatched residues. Some of these can be attributed to differences between the mouse and human p62 sequences. However, the peptide HLNLAENGL differs in its last 6 amino acids from both human and mouse p62 (M. Moran, personal communication). Because several peptides from the different digests overlapped in sequence, we show a composite representing the total peptide sequences obtained.

**METHODS.** To purify p68, about  $10^8$  c-Src<sup>Y527F</sup>-transformed NIH-3T3 cells were lysed and extracts incubated with GST-Fyn-SH3. After separation by SDS-PAGE, p68 was detected by staining with Coomassie blue and excised. Gel pieces were digested with trypsin or *Achromobacter lyticus* lysylendopeptidase in the presence of detergents and applied directly to tandem HPLC separation using a Hewlett-Packard 1090M with diode array detection (J.J.H., unpublished data). Peak fractions were sequenced using a fast-cycle automated Edman chemistry on an Applied Biosystems 477A modified as described<sup>23</sup>. The location of a phosphoserine at the second residue of the peptide DSLDPSFTHAMQLLSVEIEK was determined based on the appearance of the dehydroalanine derivative as previously described<sup>24</sup>.



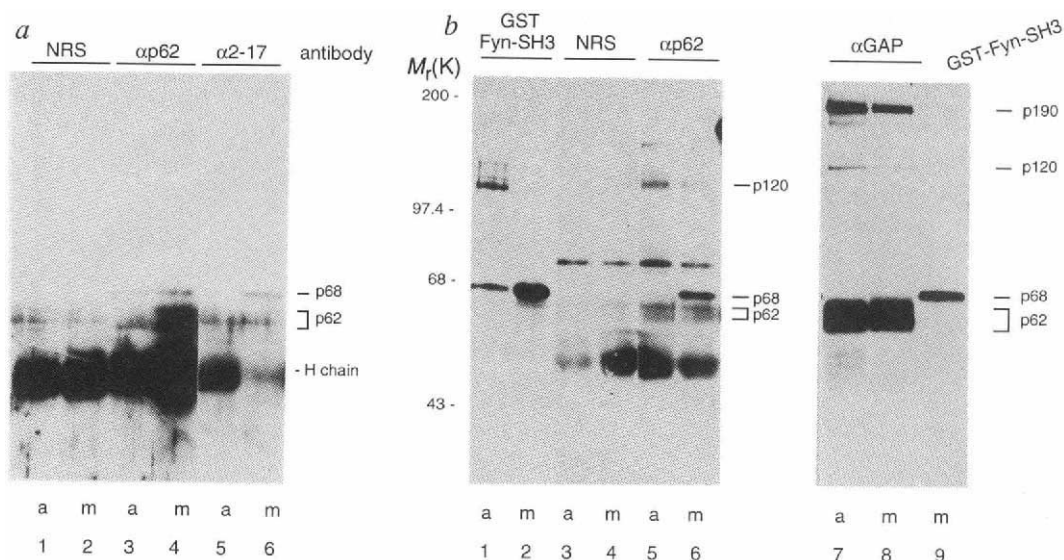


FIG. 4 p68 is antigenically related to p62 and is tyrosine-phosphorylated during mitosis. *a*, Extracts of asynchronous (lanes 1, 3 and 5) or mitotic (lanes 2, 4 and 6) c-Src<sup>Y527F</sup> cells were immunoprecipitated with normal rabbit serum (NRS, lanes 1 and 2), antibodies specific for p62 (lanes 3 and 4), or the Src-specific antibody 2-17 (lanes 5 and 6), resolved by SDS-PAGE, transferred to nitrocellulose and probed with the anti-p62 antiserum. *b*, Anti-phosphotyrosine immunoblotting was done on proteins isolated by GST-Fyn-SH3 binding (lanes 1, 2 and 9), or immunoprecipitation with normal rabbit serum (lanes 3 and 4), anti-

p62 antibodies (lanes 5 and 6) or anti-GAP antibodies (lanes 7 and 8), from asynchronous (*a*) and mitotic extracts (*m*) of c-Src<sup>Y527F</sup> cells. METHODS. Preparation of extracts, GST-SH3 binding, immunoprecipitations and anti-phosphotyrosine immunoblotting were as described. The p62 antibody was from Santa Cruz Biotech. Anti-GAP serum was a gift from T. Pawson. Sizes of prestained markers (Gibco-BRL) are indicated on the left. The positions of p62 (which runs as a doublet), p68, and proteins of 120 and 190K (which probably represent GAP and GAP-associated p190) are marked on the right.

itself did not associate with the GST-SH3 construct, when analysed by anti-phosphotyrosine immunoblotting (Fig. 4*b*) or [<sup>35</sup>S]methionine labelling (data not shown). Furthermore, p68 was not associated with GAP (Fig. 4*b*). These data confirm that c-Src associates with a 68K p62-related protein, and that this complex only exists in mitotic cells.

We describe a mitotic substrate of c-Src<sup>Y527F</sup> that could associate with the SH3 domain of Src. We also observed association of p68 with wild-type, overexpressed c-Src, and detected a tyrosine-phosphorylated, 68K, SH3 domain-binding protein in mitotic extracts of non-transformed cells (Fig. 1), suggesting that p68 becomes tyrosine-phosphorylated during mitosis in normal as well as Src-transformed cells. Because tyrosine-phosphorylated p68 can also associate with the SH2 domain of Src *in vitro*, we cannot rule out that, once phosphorylated, the association is in part mediated by the SH2 domain. Nevertheless, in preliminary experiments, p68 did not associate *in vivo* with a Src molecule lacking the SH3 domain (data not shown). The p68 protein is highly related to, but also distinct from, the GAP-associated protein p62, which is a protein that has multiple proline-rich motifs<sup>12</sup>, and therefore the potential to interact with SH3 domains<sup>14–20</sup>. The nature of the difference between p62 and p68 is not clear. Given the extensive sequence similarity observed, it is less likely that p68 is the product of a different gene, and more likely that it is the product of an alternatively spliced form of p62. In any case, p62 and p68 are functionally distinct: p62 associates with GAP in a phosphotyrosine-dependent manner<sup>13</sup>, but did not associate with SH3 domains (Fig. 4). The p68 form, in contrast, associated with the SH3 domains of Src and Fyn in a phosphotyrosine-independent manner, but did not associate with GAP. The function of p62 is not known, although it has homology to RNA-binding proteins, and can bind RNA<sup>12</sup>. It will be interesting to determine whether tyrosine phosphorylation of p68 affects this binding. Finally, unlike wild-type c-Src, c-Src<sup>Y527F</sup> does not become further activated at mitosis<sup>21</sup>, yet we

only detected the association of p68 and c-Src<sup>Y527F</sup> during mitosis. It is possible that only on nuclear envelope breakdown can Src and p68 interact. The function of mitotically activated Src is not known, but p68 may be an important substrate. □

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# Cell-cycle calcium transients driven by cyclic changes in inositol trisphosphate levels

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TRANSIENT changes in intracellular calcium ( $[Ca^{2+}]_i$ ) have been shown to punctuate the cell cycle in various types of cells in culture<sup>1–5</sup> and in early embryos<sup>6–12</sup>. The  $[Ca^{2+}]_i$  transients are correlated with cell-cycle events: pronuclear migration, nuclear envelope breakdown, the metaphase–anaphase transition of mitosis, and cytokinesis. Mitotic events can be induced by injecting calcium and prevented by injecting calcium chelators into the sea urchin embryo<sup>10,13</sup>. Cell-cycle calcium transients differ from the transients linked to membrane signal transduction pathways: they are generated by an endogenous mechanism, not by plasma membrane receptor complexes, and their trigger is unknown. We report here that the phosphoinositide messenger system oscillates during the early embryonic cell cycle in the sea urchin, leading to cyclic increases in inositol trisphosphate that trigger cell-cycle  $[Ca^{2+}]_i$  transients and mitosis by calcium release from intracellular stores.

Inositol 1,4,5-trisphosphate ( $InsP_3$ ) levels fluctuate during the first two cell cycles in the sea urchin embryo. There is an early and rapid turnover of  $InsP_3$  associated with the fertilization calcium transient that is over by 1 min after fertilization<sup>14</sup>. Looking later in the cell cycle we found that  $InsP_3$  mass doubled in 10 min (Fig. 1) to reach a peak at a time corresponding to pronuclear migration. Five minutes later it had fallen to values below those seen before fertilization. Two further waves of increased  $InsP_3$  ensued, peaking at 30 and 45 min, which were correlated with fusion of the pronuclei and nuclear envelope breakdown, respectively (50% breakdown of the nuclear envelope in the egg population occurred at ~45 min). One hour after fertilization, when most of the embryos were entering anaphase, the intracellular levels of  $InsP_3$  rose to a peak two- to threefold higher than the unfertilized level. This peak was followed by a further, smaller increase as the embryos underwent cytokinesis (the  $InsP_3$  peaks associated with mitosis are labelled 1, 2 and 3 in Fig. 1). We found this pattern of  $InsP_3$  production in three separate experiments (Fig. 1a). The three experiments were highly correlated ( $P < 0.001$ ), indicating that peaks and troughs occurred in synchrony in three different egg batches. Peaks in  $InsP_3$  levels, significantly greater than the mean level ( $P < 0.01$ ), occurred at pronuclear fusion, streak stage, nuclear envelope breakdown, anaphase and cleavage (Fig. 1b). Levels were also significantly greater during the peaks than during the troughs between. Most of the sampling variance is concentrated at the times of peak  $InsP_3$  levels (Fig. 3c), suggesting that  $InsP_3$  peaks in individual eggs are short-lived and larger and sharper than those measured in the egg population. These results show that  $InsP_3$  mass increases at times corresponding to pronuclear migration and fusion, streak stage, breakdown of the nuclear envelope, anaphase onset, and cleavage; these are the times at which  $[Ca^{2+}]_i$  transients have been reported<sup>2,9</sup>.

Cell-cycle progression is controlled by ubiquitous cyclin-dependent kinases<sup>15,16</sup>. Protein synthesis inhibitors block cyclin

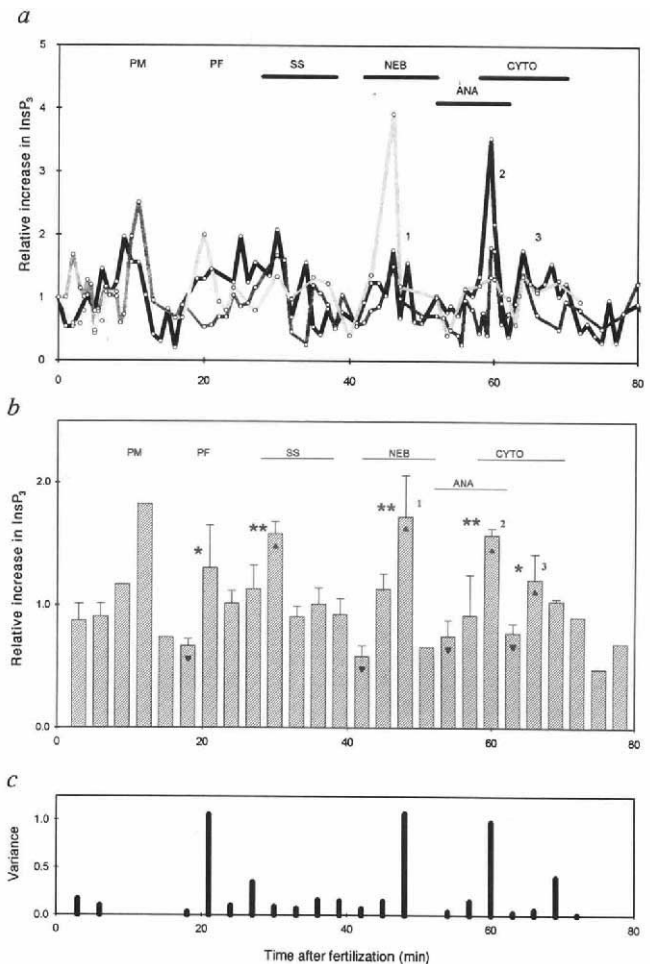


FIG. 1 Changes in  $InsP_3$  mass during the first embryonic (mitotic) cell cycles. Data are normalized to unfertilized levels (1.5, 1.22 and 0.86 pmol per mg protein in the experiments shown). PM, pronuclear migration; PF, fusion of the pronuclei; SS, streak stage; NEB, nuclear envelope breakdown; ANA, anaphase onset; CYTO, cytokinesis (cleavage). a, Three separate experiments on different egg batches. b, Mean  $InsP_3$  levels in each three minute period for each experiment were combined and analysed statistically ( $n=3$ , or  $n=2$  if no error bar). In each experiment, peak values were significantly different from non-peak levels ( $P < 0.005$ ). The three experiments are highly correlated ( $r=0.8$ ,  $0.84$  and  $0.94$  for the three cross-correlations,  $P < 0.001$  for all three, calculating  $t$  as  $r\sqrt{(n-2)/(1-r^2)}$ ). A one-tailed Student's  $t$ -test shows that the peaks at PF, SS, NEB, ANA and CYTO are significantly greater than the mean level (\*:  $P < 0.01$ ; \*\*:  $P < 0.001$ ) and that peaks at SS, NEB, ANA and CYTO (marked  $\blacktriangle$ ) are significantly ( $P < 0.05$ ) greater than the troughs (marked  $\blacktriangledown$ ). c, Sampling variance in the three experiments shown in a and b. The largest sampling variance is associated with the  $InsP_3$  peaks. This suggests that the apparent size of the peak is critically dependent on cell cycle synchrony and precise time of sampling. Time after insemination is shown in this and other figures. Fertilization occurred within 20 s. Cell cycle times were normalized to completion of cleavage at 70 min.

METHODS. Samples (*P. lividus*, 0.5 ml of a 4% v/v egg suspension, 22 °C) were taken every 1–5 min and  $InsP_3$  was measured using a binding assay (Dupont/NEN). Sample preparation and assay are described in ref. 28. Basal mass was  $1.1 \pm 0.3$  pmol per mg protein ( $0.25 \pm 0.07$   $\mu$ M, mean  $\pm$  s.e.m.,  $n=5$ ) in unfertilized eggs<sup>14</sup>. Cell-cycle events were scored in the light microscope.

synthesis and prevent cell-cycle progression in sea urchin embryos<sup>8,17</sup>. We used one inhibitor, emetine, at a blocking concentration that prevented nuclear envelope breakdown<sup>18</sup> and another, nordidemnin<sup>19</sup> at a concentration that caused embryos to arrest in prophase of the second cell cycle. In both cases (Fig. 2a) we found peaks of  $InsP_3$  production during mitosis.  $InsP_3$

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levels in the presence of either inhibitor showed autocorrelation over two cell cycles with a period that corresponded to 63 min; the cell cycle time is ~60–70 min (Fig. 2b). In each of the experiments with inhibitors,  $\text{InsP}_3$  levels were significantly ( $P < 0.01$ ) higher at times when controls were in mitosis than at times when controls were in interphase (Fig. 2b). These data indicate that the  $\text{InsP}_3$  cycles have an endogenous rhythm, with a period corresponding to the cell-cycle time, and are independent of mitotic kinase activation and of the nuclear events of mitosis itself.

We also measured turnover of phosphatidylinositol mono- and bisphosphates<sup>20</sup> ( $\text{PtdInsP}$  and  $\text{PtdInsP}_2$ ) and the amount of  $\text{PtdIns}$  over several cell cycles. Peaks of incorporation into  $\text{PtdInsP}_2$  coincided with pronuclear fusion and cleavage in the first cell cycle. Each mitosis showed a similar pattern, with an early dip giving a trough in mid-mitosis, followed by a rise, and ending with a peak at or just after cleavage (Fig. 3a, b). The pattern was also seen in embryos treated with inhibitors of protein synthesis (Fig. 3b). Similar peaks of incorporation into  $\text{PtdInsP}$  were seen during mitosis (Fig. 3c): peaks in  $\text{PtdInsP}$  incorporation corresponded to troughs in  $\text{PtdInsP}_2$  incorporation. We found cell-cycle synchronized cycles of  $\text{PtdIns}$  abundance (Fig. 3d), but with the major component at half the cell-cycle frequency. Nordidemin was used to delay the cell cycle by 25 and 100%, when the  $\text{PtdIns}$  cycles remained significantly correlated and in phase with the controls (Fig. 3d). We cannot directly relate changes in  $\text{PtdInsP}_2$  and  $\text{PtdInsP}$  turnover to the  $\text{InsP}_3$  transients, but it is striking that these other components of the phosphoinositide messenger pathway show a cyclic variation that is apparently independent of activation of the mitotic kinase.

We tested for the involvement of  $\text{InsP}_3$  receptors in the mitotic calcium transients by using the  $\text{InsP}_3$  antagonist heparin<sup>21,22</sup>. In control eggs, 100–150 nM  $[\text{Ca}^{2+}]_i$  transients precede nuclear envelope breakdown and anaphase onset and a broad 200 nM transient accompanies cleavage (labelled 1, 2 and 3 in Fig. 4a; compare Fig. 1), coinciding with the peaks of  $\text{InsP}_3$  production (Fig. 1). In eggs microinjected with heparin, these transients were absent and the embryos failed to undergo mitosis (Fig. 4b), indicating that the  $[\text{Ca}^{2+}]_i$  transients were generated by  $\text{InsP}_3$ . In emetine-blocked eggs there were marked futile calcium oscillations (Fig. 4c). The futile  $[\text{Ca}^{2+}]_i$  oscillations may mirror the periodic increases in  $\text{InsP}_3$  seen with the protein synthesis inhibitors (Fig. 2), but we cannot be certain as we do not know the phase relations of the  $[\text{Ca}^{2+}]_i$  oscillations in individual eggs.

**FIG. 2** The effects of protein synthesis inhibitors on  $\text{InsP}_3$  levels during the cell cycle. **a**, Top, controls. Two experiments over two cell cycles are shown. The time of mitosis (from 50% NEB to 50% cleavage) is indicated. Middle, the same egg batches bathed in sea water containing 16  $\mu\text{M}$  of the protein synthesis inhibitor nordidemin for 15 min before fertilization. This treatment does not block first mitosis (Fig. 3d). Bottom, the same egg batches bathed in sea water containing 100  $\mu\text{M}$  of the protein synthesis inhibitor emetine. To indicate episodes of high  $\text{InsP}_3$  levels, data points lying above the 95% confidence interval of the mean (assuming a normal distribution) for the  $\text{InsP}_3$  values measured after treatment with inhibitors are shown as asterisks. **b**, Statistical analysis of variations in  $\text{InsP}_3$  levels after treatment with inhibitors (left, nordidemin; right, emetine). Top, correlograms showing the variation in the autocorrelation coefficient ( $r(k) = \sum_{t=1}^{N-k} [x_t - \bar{x}] \cdot [x_{t+k} - \bar{x}] / \sum_{t=1}^N [x_t - \bar{x}]^2$ )<sup>29</sup> with lag  $k$  for both experiments with each inhibitor. There is a marked periodicity in the correlogram. The fitted curve has a period of 63 min, indicating that  $\text{InsP}_3$  levels vary with a strong component at a frequency corresponding to the cell division cycle. Bottom, distribution of  $\text{InsP}_3$  levels during the cell cycle. Open bars show distribution of levels during times corresponding to interphase in controls and filled bars to mitosis. In each experiment,  $\text{InsP}_3$  levels are consistently higher during the mitotic period: the biggest peaks of  $\text{InsP}_3$  occur during this period and a t-test (one-tailed) indicates that mean  $\text{InsP}_3$  concentrations are significantly ( $P < 0.01$ ) higher during the mitotic period than during interphase.

Our data suggest that an endogenous oscillator that is independent of the cyclin oscillator<sup>15,17</sup> controls the production of  $\text{InsP}_3$  at key points in the cell cycle and that  $\text{InsP}_3$  generates the cell cycle  $[\text{Ca}^{2+}]_i$  transients that appear to control the timing of mitosis onset (nuclear envelope breakdown) in early sea urchin embryos. Comparison of the shapes of both  $\text{InsP}_3$  and  $[\text{Ca}^{2+}]_i$  transients in control and kinase-inhibited embryos sug-

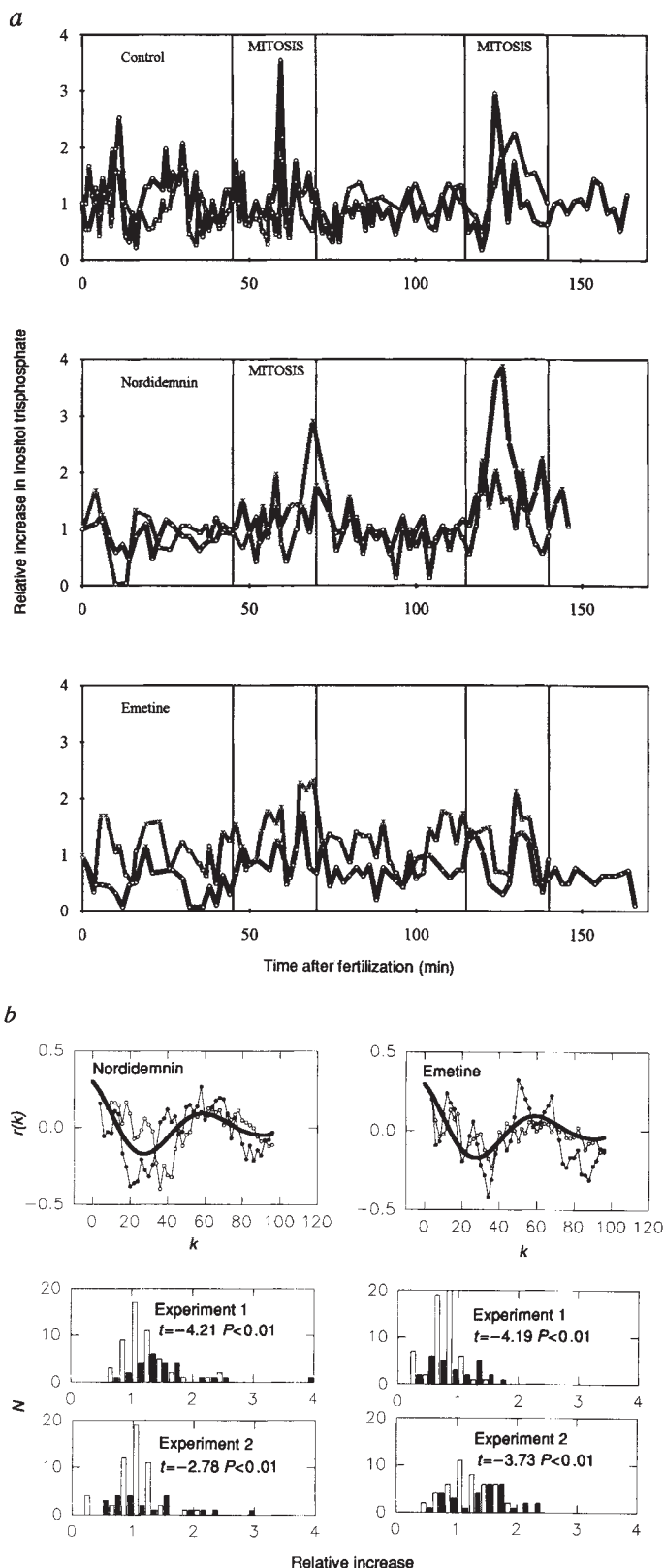
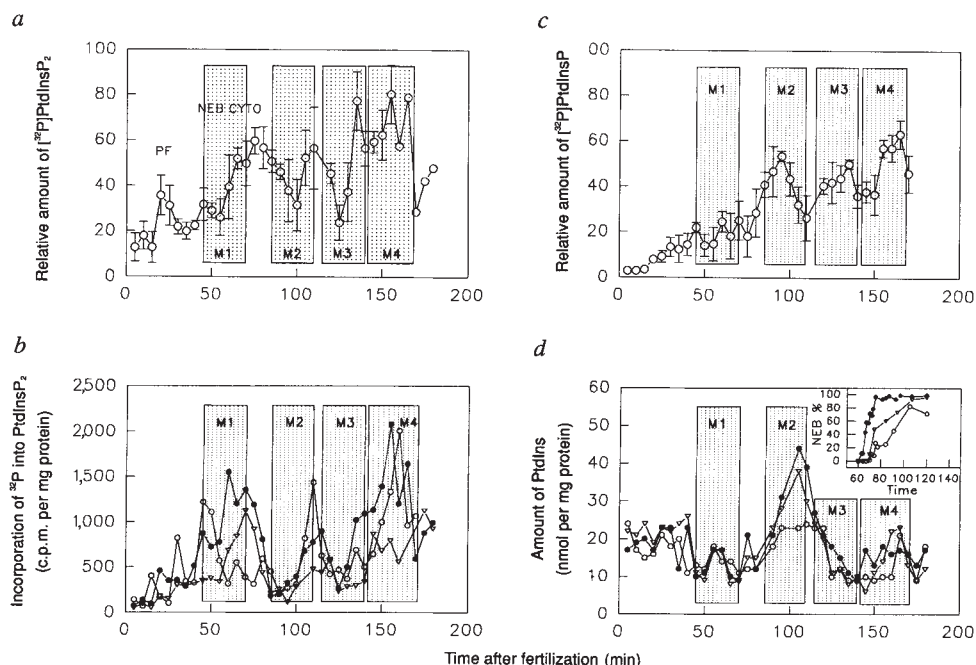


FIG. 3 Incorporation of  $^{32}\text{P}$  into PtdInsP<sub>2</sub> and PtdInsP during the first four embryonic cell cycles. Shaded bars indicate the periods when the control embryos were in mitosis (M1 is first mitosis, M2, second mitosis, and so on). *a* and *b*, PtdInsP<sub>2</sub>; *c*, PtdInsP; and *d*, PtdIns: *a* and *c* are the means  $\pm$  s.e.m. of three experiments on three separate egg batches; *b* compares the effects of the protein synthesis inhibitors emetine (100  $\mu\text{M}$ ;  $\circ$ ) or nordidemnin (16  $\mu\text{M}$ ; 15 min preincubation;  $\nabla$ ) with controls ( $\bullet$ ) in a single egg batch (one of two experiments). Treatment with either inhibitor led to inhibition of more than 99% of protein synthesis, as judged by rate of incorporation of [ $^{35}\text{S}$ ]methionine into protein over 175 min (rates of incorporation, determined by regression over 34 samples taken every 5 min, were: control,  $1.9\% \text{ min}^{-1}$ ; emetine,  $0.002\% \text{ min}^{-1}$ ; nordidemnin,  $0.000\% \text{ min}^{-1}$ ). Cycles of PtdInsP<sub>2</sub> production persist in the presence of inhibitory concentrations of emetine and nordidemnin (*b*). The PtdInsP<sub>2</sub> cycles seen after treatment with either inhibitor are highly correlated ( $r=0.5$  and  $0.8$ , respectively, for emetine and nordidemnin, with  $n=32$  and  $P<0.01$  for both, calculating  $t$  as  $r/\sqrt{(n-2)/(1-r^2)}$ ). Cyclic variation in PtdIns in controls ( $\bullet$ ) was unaffected by delaying the cell cycle using nordidemnin (*d*); *d*, inset, shows the cell-cycle delay induced by preincubation for 15 ( $\circ$ ) or 30 ( $\nabla$ ) min with 16  $\mu\text{M}$  nordidemnin. The PtdIns cycles seen after treatment with inhibitor are also highly correlated ( $r=0.9$  and  $0.6$ , respectively, for 15 and 30 min treatment, with  $n=34$  and  $P<0.01$  for both, calculating  $t$  as  $r/\sqrt{(n-2)/(1-r^2)}$ ).

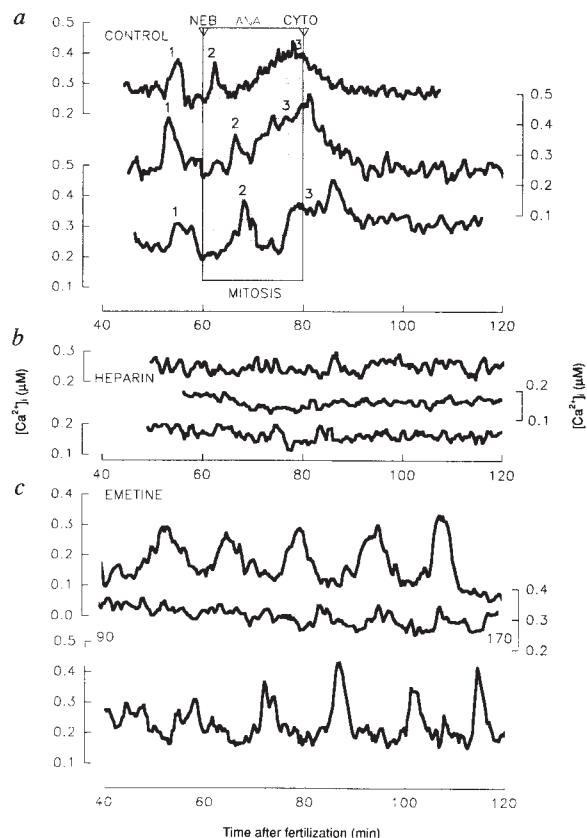
METHODS. Carrier-free  $^{32}\text{P}$ -orthophosphate (40  $\mu\text{Ci ml}^{-1}$ ) was added to



eggs just before fertilization. Samples (1 ml of 4% v/v egg suspension) were taken at 1–2 min intervals, centrifuged, lipids extracted into chloroform/methanol/HCl and quantified by thin layer chromatography and liquid scintillation counting as described previously<sup>20</sup>. Under these nonequilibrium conditions,  $^{32}\text{P}$ -orthophosphate continues to accumulate in the cytoplasm during the course of the experiment, but there is no evidence of a cyclic accumulation pattern (not shown). Data from three experiments were normalized as described for Fig. 1 and by taking the most heavily labelled sample in each experiment as 100%. PtdIns was measured using a phosphate assay after chromatographic purification and mineralization<sup>28</sup>.

FIG. 4 Mitosis-associated cytoplasmic calcium transients in single sea urchin embryos during the first cell cycle. *a*, Controls: NEB is preceded by a 100–150 nM [ $\text{Ca}^{2+}$ ], transient ( $85 \pm 8$  nM, mean  $\pm$  s.e.m.,  $n=23$ ) lasting 2–3 min (peak 1); a second transient of similar magnitude and duration occurs before anaphase (peak 2); cleavage is preceded and accompanied by a slightly larger, longer-lasting transient (peak 3). *b*, After microinjection of the InsP<sub>3</sub>-antagonist heparin (200  $\mu\text{g ml}^{-1}$ ), [ $\text{Ca}^{2+}$ ] fluctuates over a 50 nM range, but no transients are observed. The embryos do not enter mitosis, indicating that blocking the InsP<sub>3</sub>R and [ $\text{Ca}^{2+}$ ] release prevents entry into mitosis (NEB). As mitosis is blocked at entry, it is unclear from these experiments whether blocking the anaphase-associated calcium transient would block anaphase onset. Controls were injected with the same concentration of desulphated heparin. *c*, Embryos treated with the protein synthesis inhibitor emetine (at 100  $\mu\text{M}$ , which blocks 99% of protein synthesis in this and related species<sup>17,26</sup>) do not undergo mitosis, but show marked 100–200 nM [ $\text{Ca}^{2+}$ ] oscillations at the time when untreated embryos are in mitosis that continue for tens of minutes.

METHODS. *Lytechinus pictus* embryos were kept in sea water at 16 °C on a microscope stage and microinjected 40 min after fertilization with the calcium indicator Fura-2-dextran (we find the dextran-conjugated Fura-2 to be better at detecting small [ $\text{Ca}^{2+}$ ] transients than Fura-2 itself); [ $\text{Ca}^{2+}$ ] was determined using a fluorescence ratio method<sup>9,13</sup>. When necessary, heparin or desulphated heparin (100 mg  $\text{ml}^{-1}$ ) were coinjected with the indicator dye. Three experiments representative of at least eight experiments for each condition are shown. In *a*, NEB was normalized to 60 min after fertilization, the mean time to NEB at 16 °C. Thus, NEB occurs later than in Fig. 1, where, at 22 °C, NEB occurs at 45 min. Note also that here observations on individual eggs are shown, whereas the data in Figs 1–3 are obtained from egg populations.





gests that activation of the mitotic kinase may shape the waveform of the endogenous oscillator to generate sharp spikes of  $\text{InsP}_3$  and  $[\text{Ca}^{2+}]_i$  during mitosis. This is an interesting example of rapid, periodic activation of the phosphoinositide messenger system to cause  $\text{InsP}_3$  production that is independent of an extracellular agonist. It is possible that  $\text{InsP}_3$  production is linked to the centrosome cycle, because this can also be uncoupled from mitosis<sup>23,24</sup>. There are indications that these data may be of general application, as an anti-Ptd $\text{InsP}_2$  antibody can arrest the cell cycle in frog embryos<sup>25</sup> and in yeast<sup>26</sup> and  $\text{InsP}_3$  levels increase at cleavage<sup>27</sup> in the frog. □

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